BRAIN DIVERSITY DEVELOPS EARLY: A STUDY ON THE ROLE OF PATTERNING ON VERTEBRATE BRAIN EVOLUTION

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A STUDY ON THE ROLE OF PATTERNING ON VERTEBRATE BRAIN EVOLUTION

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DV	dorsal/ventral axis
AP	anterior/posterior axis
ANR	anterior neural ridge
MHB	midbrain-hindbrain boundary
PSB	pallial-subpallial boundary
ZLI	zona limitans intrathalamica
Hh	Hedgehog
WNT	Wingless
FGF	Fibroblast Growth Factor
BMP	Bone Morphogenic Protein
LF	*Labeotropheus fuelleborni*
MZ	*Maylandia zebra*
CA	*Cynotilapia afra*
AJ	*Aulonocara jacobfreibergeii*
CB	*Copadichromis borleyi*
MC	*Mchenga conophorus*
LiCl	Lithium Chloride
DMSO	Dimethyl Sulfoxide

SUMMARY

The brain has been one of the central foci in studies of vertebrate evolution. Recent studies on brain evolution have focused on differential neurogenesis as the driving cause behind brain diversification. However, neurogenesis is a relatively late event in brain ontogeny. The role of earlier events in patterning in brain evolution is difficult to examine in model organisms (mouse, chick, frog, zebrafish) because of long evolutionary divergence times, which result in large differences in brain development and morphology.

Work in East African cichlids and other emerging fish models like the Mexican cavefish (*Astyanax mexicanus*) offer new insight on the role of patterning on brain evolution. Both cichlids and cavefish have large, ecologically relevant changes in brain morphology but little difference in the underlying genetic pathways that control development. These fish can be grouped into two major catagories according to habitat; for cichlids it is rock-dwelling (known locally as mbuna) and sand-dwelling (non-mbuna) lineages and *Astyanax* have a eyed surface morph and a cave-dwelling eyeless form. The brain development of mbuna versus non-mbuna is defined by changes in gene deployment working along the dorsal/ventral (DV) and anterior/posterior (AP) neuraxes, respectively. Thus, comparison of disparate fish ecotypes offer a new perspective of the role of patterning on brain evolution; through the slight and early modification of signal

pathways working across 3-D axes, and a subsequent magnifying effect across ontogeny, evolution can generate widespread changes in the brain.

To illustrate this patterning model of brain evolution, two comparative studies were done between mbuna and non-mbuna, examining the action of the Wingless (WNT) pathway across the AP axis and the Hedgehog (Hh) pathway along the DV axis. The first study found that non-mbuna cichlids have a more rapid expansion of WNTs from the MHB into the presumptive midbrain and diencephalon versus mbuna. This leads to a shift in the zona limitans intrathalamica (ZLI), a boundary that splits the diencephalon, and a correlated expansion of the thalamus. This brain structure is involved in sight processing and could be of ecological benefit to vision-focused non-mbuna. In support of this observation, a non-synonomous amino acid change was found in the *irx1* gene, which is upregulated by WNTs and helps position the ZLI. This SNP is alternatively fixed between mbuna and non-mbuna, which indicates evolutionary significance in the aforementioned difference in ZLI placement.

The second study described a difference in the position of another boundary, the pallial-subpallial boundary (PSB), which forms within the developing telencephalon. The PSB splits the telencephalon in to the pallium, which processes visual signals, and the subpallium, which develops into the olfactory bulbs. Mbuna possess a larger subpallium and a dorsal shift of the PSB relative to non-mbuna, which have a larger pallium. The shift in PSB position is correlated to an early expansion of *shh* dorsally in mbuna relatively to non-mbuna, which causes the subpallial gene *foxg1* to come up more quickly in

mbuna and retard the progress of WNTs into the telencephalon. Interestingly, small molecule-based manipulation of both the Hh and WNT pathways revealed that the action of these pathways on the telencephalon is tightly integrated; manipulation of one pathway affects the other. This could be due to the mediation activity of *foxg1* and another gene, *gli3*, which are downstream targets of the Hh and WNT pathways, respectively. This results in larger subpallia for mbuna, and larger pallia for non-mbuna, which can be correlated to previously described differences in adult cichlid brains.

Overall, East African cichlids are an excellent system to investigate the role of patterning on brain evolution because they allow for the comparison of the earliest patterning events in brain ontogeny between distinct ecotypes. Combined with comparative studies in *Astyanax mexicanus*, scientists can use the information they generate to shed new light on brain evolution. These fish systems link the many years of study in brain development of model systems to the brain morphology comparisons employed in classic studies of brain evolution. Only when researchers look at the full scope of ontogeny, examining both patterning and neurogenesis, will we gain insight on vertebrate brain evolution

CHAPTER 1

INTRODUCTION

Vertebrate brain evolution has always been a subject of intense interest, due to mankind's own evolutionary history. Vertebrate brain morphology is as diverse as the habitats they occupy around the world. Much of the basis for recent study on brain evolution began in the mid 90s, with the work of Finlay and others on evolutionary trends in the brain across mammalian taxa (Finlay and Darlington 1995). Finlay examined a brain dataset generated by Stephan and colleagues that measured both total brain size and the individual structures within the brain, then compared them to total body size; this was repeated in many species across taxa representing mammalian diversity (Stephan *et al.* 1981). Finlay *et al.* (1995, 2001) found that the brain tends to increase in size relative to body size with the exception of the neocortex, a part of the telencephalon that increases almost exponentially in relation to body size, especially in the primate lineage (Finlay and Darlington 1995). Most importantly, this study demonstrated that the diversity of function and form in vertebrate brain morphology can be encompassed in a general, overarching model.

The exponential increase of the neocortex was linked to a tightly controlled process known as neurogenesis, which regulates size, layout, and neuron number in the cortex; first described in investigations in the macaque

monkey in the late 80s (Rakic 1988). Neurogenesis is responsible for the maturation of undifferentiated neuronal precursor cells into fully functional mature neurons. An important aspect of neurogenesis is that as the cells mature from precursor to neuron, they become post-mitotic, e.g. lose their ability to replicate (Rakic 1988). Finlay and others suggested that the driving factor behind the rapid increase in neocortex size was due to a delay in the final stages of neurogenesis. Precursors in the cortex mature in temporally staggered waves, or 'rounds.' If there is a delay between rounds, this gives the precursors more time to replicate so that there are more cells available to mature upon receiving an appropriate signal. This also gives precursors fated to mature in later rounds more time as well, resulting in the same exponential increase observed across mammalian taxa (Caviness *et al.* 1995, Rakic 1995, Takahashi 1996). This theory became known as 'late equals large;' the theory was tested in both rodents and primate cortex development and found that it sufficiently explained the difference in size and layout between rodents and primates (Takahashi 1996, Darlington 1999, Clancy *et al.* 2001). Thus neurogenesis became the overarching model that linked the differences in brain morphology from fish to reptiles to mammals.

Around the turn of the century there were several more fine scale analyses done on the dataset generated by Stephan *et al.*, which investigated individual brain structures and their trends across mammalian taxa. Researchers found that in general, brain structures grow together as the entire brain grows (Clark et al 2001), but there was also evidence of a 'trade-off' (meaning as one structure grows across taxa, another shrinks) between structures of the brain

adjacent to the neocortex in primates (Barton *et al.* 2000) as well as evidence of piecemeal increases in size of some structures relative to others (de Winter *et al.* 2001). This led to a refinement of the late equals large theory to include two competing, yet non-mutually exclusive models: developmental constraint and mosaic evolution (Finlay *et al.* 2001). Developmental constraint makes the assumption that brain development is too important to be modified, and evolutionary changes would be under purifying selection. Thus any evolutionary change that is fixed would most likely affect the entire brain as a whole (Clark *et al.* 2001, Finlay *et al.* 2001) Mosaic evolution allows for the possibility that an evolutionary change can act within a brain structure, which would affect only that structure (Barton *et al* 2000). However, it was considered that a likely place for such a change to occur would be during neurogenesis, since this has already been observed in the neocortex of primates (Finlay *et al.* 2001).

A notable study by Chenn *et al.* found a developmental link to the late equals large theory and the two models of developmental constraint and mosaic evolution when they discovered the role of the Wingless (WNT) pathway in regulating the size of the neocortex in mice (Chenn *et al*. 2002). They were able to find that an effector of the WNT pathway, β-catenin, when upregulated can increase the size of the mouse neocortex by delaying the onset of neurogenesis, transforming it very much the same way as evolution has. This link to development opened new avenues of study in brain evolution, using model organisms (mouse, chicken, frog, zebrafish) in an attempt to further understand how brain evolution works through development to generate brain diversity

(Striedter 1997, Wullimann and Mueller 2004, Striedter 2005, Mueller and Wullimann 2009, Yopak *et al.* 2010). However because of the constraint model, the large amount of evidence in favor of neurogenesis as the primary cause, and the large ontogenetic, morphological, and phylogenetic differences between model organisms, it was difficult to investigate the role of developmental events prior to neurogenesis in brain evolution until recently.

This dissertation will attempt to elucidate and examine the role of early events in the establishment of diversity of the brain. The classic model systems have been used for decades to elucidate how these events set up the brain, but by their nature cannot link these events to vertebrate brain evolution. To do this, this study uses comparisons between closely related species of fish, primarily within the cichlids of Lake Malawi, to offer a new approach to brain evo-devo. These fish exhibit a large amount of brain diversity on a background of genomic similarity, allowing experimentation that is not possible in the classic model systems. This allows for a new way to investigate brain evolution, which does not focus on late-acting neurogenesis as a cause, but instead examines all of brain ontogeny.

In chapter 2, I present a new, patterning-focused way to examine brain evolution: by looking at the establishment of the three-dimensional axes of the neural tube that will give rise to the brain. This represents the earliest events in brain ontogeny, which have only recently been investigated in terms of brain evolution. I postulate that change to these axes can drive brain diversity; if the change is early, this change is across many structures within the brain. However,

as the brain begins to develop signal-emitting boundaries that subdivide the tube, the effect of change becomes limited to discrete areas of the brain. This allows for the brain to change rapidly in response to selective pressures. I give evidence in cichlids and other fishes that changes in early patterning is a natural alternative to the neurogenesis-focused approach to brain evolution.

Chapters 3 and 4 will illustrate how the model presented in chapter 2 can be use to examine how diversity is achieved in the Lake Malawi cichlids. The chapters investigate the deployment of major gene pathways (Wingless and Hedgehog) working across the anterior/posterior (AP) and dorsal/ventral (DV) axes, respectively. Each study looks at two ecologically distinct groups in Lake Malawi, the rock-dwellers (mbuna) and sand-dwellers (non-mbuna), which possess different brains, but share the same genetic background. Chapter 3 shows that an early difference in WNTs acting along the AP is responsible for a shift in an important signaling boundary, the zona limitan intrathalamica (ZLI) and the elaboration of a sight processing forebrain structure in non-mbuna. This is an example of an early change which changes the layout of the entire brain. Chapter 4 describes a similar difference in the Hh pathway working on the DV axis, which drives the expansion of the ventral portion of the telencephalon by shifting the pallial-subpallial boundary (PSB) in mbuna; this is representative of a later change in ontogeny that targets a discrete part of the brain.

Chapter 5 restates the ultimate goal of this dissertation: to describe how early patterning events can contribute to vertebrate brain evolution. I summarize the information presented in the previous chapters and explain how

they elucidate and serve as evidence for the patterning model of brain evolution. This dissertation is not attempting to supplant neurogenesis as the dominant factor behind brain variation but instead I seek to augment our understanding of brain evolution by incorporating patterning. Indeed, brain development is a continuum of patterning to neurogenesis and neither can be examined without investigating the other. With the establishment of cichlid fishes and other similar systems as models for brain evo-devo, researchers can finally look at the entirety of ontogeny in order to answer questions integral to vertebrate brain evolution.

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CHAPTER 2

INTEGRATED BRAIN DIVERSITY ALONG THE EARLY NEURAXES[1]

2.1 Abstract

Brains develop under the influence of signaling centers that link major dorsal/ventral (DV) and anterior/posterior (AP) axes. Over ontogeny, these 'developmental neuraxes' progress from near global signaling gradients into more localized gene expression domains, separated by molecular boundaries. Therefore, developmental changes along a neuraxis can have major consequences across the brain, or more precise effects on a specific structure, depending upon the time during ontogeny in which change occurs. It is well known from mammalian systems how evolution has acted later in development, via differential neurogenesis, to reshape the cortex. Recent studies in fishes show how early changes in DV and AP patterning result in divergence of integrated brain regions that ultimately define visual *vs*. olfactory ecotypes. We explore the generality of this trend and suggest that such early developmental differences integrating brain diversification along the neuraxes may be a common theme in vertebrates. Early differences in brain patterning among species imply that adult variation in sensory function and behavior manifest in the embryo.

[1] Sylvester JB, Pottin K, Streelman JT (2011) Integrated brain diversity along the early neuraxes. *Brain Behavior and Evolution* In press.

2.2 Introduction

Many of the current studies in brain evolution are rooted in comparisons across a variety of mammalian vertebrates (Stephan *et al.* 1981, Finlay *et al.* 1995). These comparisons have revealed considerable variation in the size, complexity, and layout of brains across broad phylogenetic groups. Although recent studies have added fishes, birds, and a greater diversity of mammals, a central conclusion applies: total brain size accounts for greater than 90% of the observed variation in the size of individual brain structures (Finlay *et al.* 1995, Kotrschal 1998, Barton and Harvey 2000, Finlay and Darlington 2001, Iwianuk and Hurd 2005, Gonzalez-Voyer *et al*. 2009a, Yopak *et al.* 2010). In an evolutionary sense then, vertebrates tend to expand the entire brain in response to selection pressure for any specific function. However, more fine-scale analysis shows that specific brain regions can exhibit positive or negative allometric growth relative to total size in specific vertebrate lineages (Clark *et al.* 2001, deWinter *et al.* 2001, Finlay and Darlington 2001, Reep *et al.* 2007*,* Yopak *et al.* 2010). For example, cetaceans and bats have expanded their cerebellum relative to total brain size (deWinter *et al.* 2001), several avian and sauropsid lineages have expanded the tectum (Iwianuk and Hurd 2005, Charvet *et al.* 2010b), while primates show a negative correlation between cerebral size and the limbic system (Reep *et al*. 2007).

These two major trends in brain evolution; i) total brain size accounts for most observed variation and ii) certain lineages exhibit allometric growth in

specific structures of the brain, have led scientists to postulate two, non mutually exclusive ways that brains have evolved: developmental constraint and mosaic evolution. The idea of developmental constraint is that because brain structures are highly integrated and arise from highly conserved gene networks, the path of least resistance for the brain to respond to different selection pressures is to grow the brain as a whole (Finlay *et al.* 1995, 2001). Mosaic evolution describes how vertebrate lineages target specific brain structures in response to certain demands. Proponents of this model argue that brain tissue is metabolically demanding, and therefore organisms may gain an evolutionary advantage by limiting relative growth to relevant structures (Dunbar and Shultz 2007).

While there has been much discussion regarding the prevalence and significance of developmental constraint versus mosaic evolution in the vertebrate brain literature, several studies have demonstrated that both of the observed trends can be explained via differential neurogenesis (Finlay *et al.* 2001, Fish *et al.* 2008). Neurogenesis is the process that results in functional, non-mitotic neurons from a general population of undifferentiated, self-replicating neuronal precursors. It is temporally sequential in differentiation; the earliest round of neurogenesis gives rise to precursors that can serve any function in the brain and each subsequent round gives more differentiated, functionally distinct cells (Rakic 1988, Caviness *et al.* 1995). Therefore, in order to make the entire brain, or any specific brain structure larger, neurogenesis is delayed – the longer the delay, the longer the precursors can divide prior to terminal differentiation. This delay can either cause all structures of the brain to evolve in a concerted

fashion under the constraint model, or neurogenic timing can be modified within a specific brain structure according to the mosaic model (Caviness *et al*. 1995, Rakic 1995, Barton *et al.* 2000, Finlay *et al.* 2001). Since neurogenesis can explain both constraint and mosaic models of brain evolution, there have been a variety of analyses that examine when and where neurogenesis can be 'tweaked' to give rise to the observed brain differences among mammals (Takahashi *et al.* 1996, Darlington *et al.* 1999, Clancy *et al.* 2001, Fish *et al* 2008).

However, when considering the entire span of brain development, the onset of neurogenesis is not the earliest event in ontogeny. Well prior to, and coincident with neurogenesis, presumptive neural tissue undergoes the process of patterning, or the establishment of major anterior/posterior (AP) and dorsal/ventral (DV) axes, as well as the development of important signaling boundaries (Kiecker and Lumsden 2005). Although brain development is a continuum of patterning to neurogenesis, rarely is patterning discussed as a mechanism of brain evolution. In this article, we highlight the role of neural patterning in brain evolution. We argue that early patterning changes can have significant cumulative effects across many brain structures, or more targeted effects on one structure, depending upon the timing of change. Recent studies in fishes have shown that changes in the earliest patterning networks define the difference between visual versus olfactory ecotypes in closely related populations and species (Menuet *et al.* 2007, Rétaux *et al*. 2008, Sylvester *et al.* 2010, Pottin *et al.* 2011). These results and studies in other vertebrate systems demonstrate how variation in patterning can influence brain evolution before neurogenesis

begins. In fact, because patterning precedes neurogenesis, small modifications in neural patterning may prefigure later, and larger-scale differences in the timing of neurogenesis. Reconciling patterning with neurogenesis reveals that evolution can act at any point in time during ontogeny to shape the brain.

2.3 An Overview of Vertebrate Brain Patterning

In this section, we refer to studies of a small number of laboratory model systems like the chick, frog, zebrafish and mouse. Other species may depart from the specific timing of developmental events portrayed here, but rarely from the general sequence. We describe developmental events in a generalized vertebrate model, but acknowledge the complexity that underlies our broad description of brain patterning. The vertebrate brain begins as a field of epithelial cells specified during gastrulation (Rhinn *et al*. 2006). This neural plate is induced from the dorsal mesoderm, in the margin to the adjacent epidermis, through the repression of bone morphogenetic protein (BMP) signals (Figure 2.1; (Wilson *et al*. 1997; Barth *et al*. 1999)). Signals from the margin itself, including wingless molecules (WNTs), fibroblast growth factors (FGFs) and retinoic acid, polarize the neural plate and specify posterior fate along the anterior/posterior (AP) axis. At the opposite end along the leading edge of the plate, the anterior neural ridge (ANR in fish, anterior neural boundary in other vertebrates) secretes WNT antagonists called sFRPs (secreted Frizzled-Related Proteins); a notable example is the gene *tlc.* In time, these WNT antagonists form an opposing

anterior signaling center (Figure 2.1A, 2.2A, 2.3A; (Rallu *et al*. 2002; Houart *et al*. 2002, Wilson and Houart 2004)). Nearly simultaneously, the posterior WNT gradient at its highest concentration leads to the formation of the midbrain hindbrain boundary (MHB), which compartmentalizes the neural plate into a posterior presumptive hindbrain and an anterior presumptive fore + midbrain called the prosencephalon ((Rhinn *et al*. 2005); Figure 2.2A).

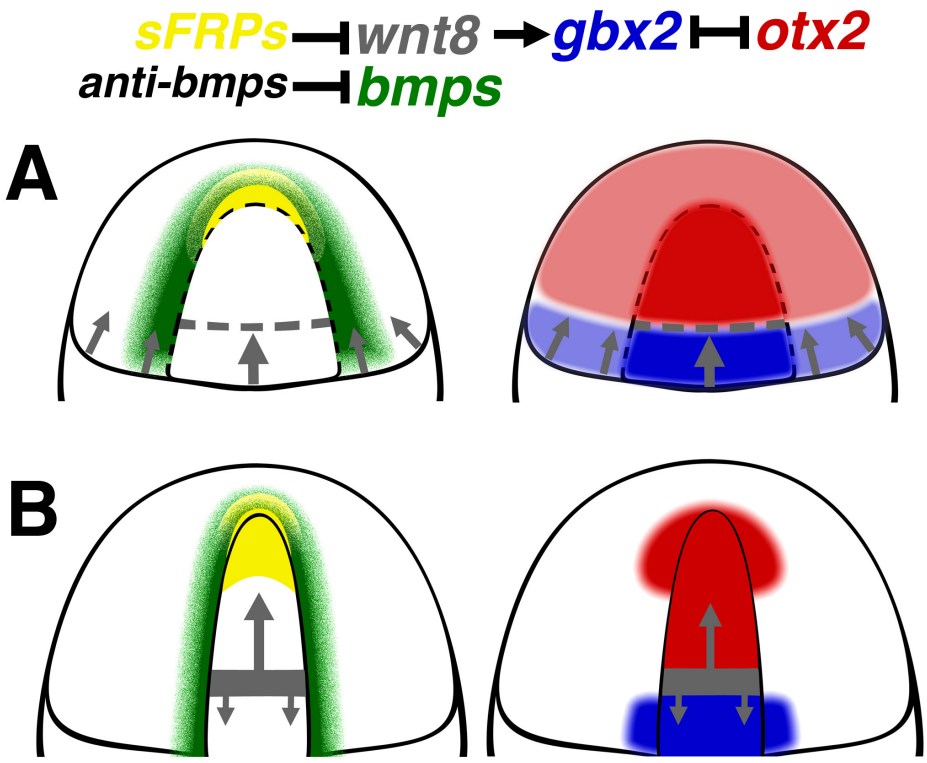

Figure 2.1 Neural Induction and Polarity Are Set During Gastrulation. A During gastrula, the neural plate is induced in a local region of BMP repression (by factors like Noggin, Chordin and Follistatin). As the neural plate induces from posterior to anterior, its leading edge does so through territory also low in BMP signal. Concurrently, WNT signals (*wnt8*) emanating from the dorsal mesoderm margin act to posteriorize the neural plate, kept in check via the action of anti-WNT signals like *tlc* and other sFRPs (left panel). *wnt8* activates the transcription factor *gbx2* in the posterior portion of the developing neural plate, *gbx2* antagonizes the anterior-specifying transcription factor *otx2* and the MHB will form at the interface of these two antagonists (right panel). **B** As development proceeds into neurula, the plate narrows into a tube, flanked by BMP-positive cells. *otx2* expression narrows as well, to the presumptive fore- + midbrain and continues to antagonize *gbx2* at the MHB (right panel). The MHB has coalesced into a discrete compartment, and sends signal into both the newly formed hindbrain and prosencephalon. The anterior progress of these signals is halted by both *tlc* and *tlc*-activated downstream transcription factors (left panel). Colors in the gene pathway correspond to colors shown in the figure panels. Gene expression patterns and neural plate size correspond to Malawi cichlid fish development.

The ANR and MHB play tug-of-war along the AP neuraxis. The MHB (i) is now the source of all posterior signals in the prosencephalon, (ii) blocks any signal from the hindbrain, and (iii) induces the region of the prosencephalon immediately anterior to the MHB to become midbrain. The ANR induces the forebrain; which consists of a combined telencephalon, eye field and diencephalon (Figure 2.2A). As gastrulation proceeds, transcription factors reinforce the newly established signaling centers. *tlc*-activated *six3* from the ANR prevents WNT signals from caudalizing the forebrain (Lavado *et al*. 2008), while *otx2* and *gbx2* set the final position of the MHB (Figure 2.1; (Li and Joyner 2001)). Additionally, transcription factors begin to transform the signal-established domains into discrete compartments that prefigure adult structures. For this reason, signaling centers like the ANR and MHB are known as secondary organizers; they facilitate independent trajectories of adjacent compartments via the integration of transcription factors along the AP neuraxis (Figure 2.2).

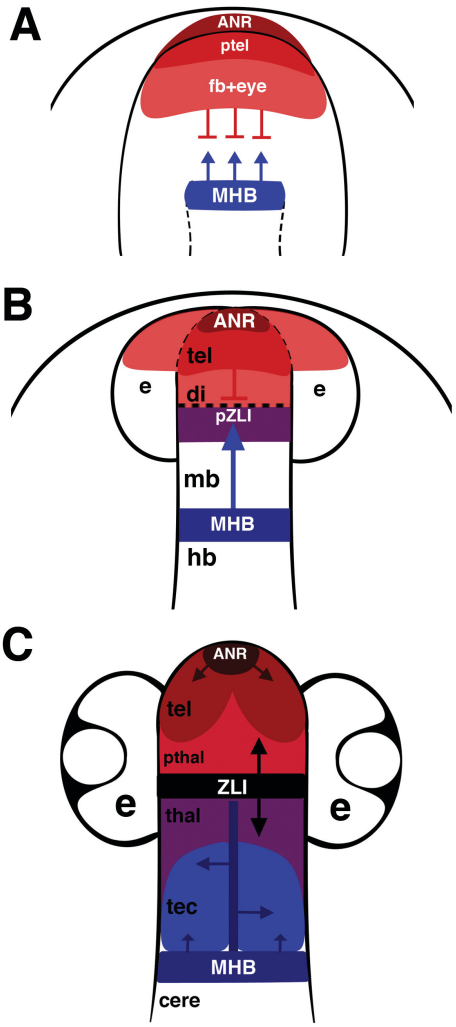

Figure 2.2 Brain Development Proceeds from General Gradients to Specialized Compartments. A Dorsal view of a generalized vertebrate embryo during late gastrula. At this stage of development, the brain is under the influence of two mutually antagonistic signaling centers, the ANR (in red) and the MHB (in blue). Signals like *tlc* and other WNT-antagonists are required to specify anterior neural tissue, which will give rise to the forebrain+eye. As the ANR influence wanes, WNT signals caudalize the posterior portions of the presumptive neural tube, which will give rise to the rest of the brain. **B** A representation of the vertebrate embryo at the neurula stage. Continuous signaling from the ANR, as well as the actions of transcription factors like *six3*, *rx*, and *pax6*, have split the eyes (e) from the presumptive telencephalon (tel) and diencephalon (di). Eye development will eventually cease to be under the influence of the ANR and continue on its own developmental trajectory. The MHB differentiates the midbrain (mb) and hindbrain (hb), and a strong push in WNT signal, represented by the blue arrow, moves into the posterior diencephalon (di, purple), which receives input from both the ANR and MHB. The actions of several transcription factors, activated by the ANR anterior and WNT signals posterior, drive the formation of a third signaling boundary, the zona limitans intrathalamica (pZLI, dotted line). **C** As the vertebrate embryo proceeds into somitogenesis, the ANR, ZLI, and MHB no longer signal across the entire brain, but in localized compartments; this represents their secondary organizer activity. The actions of these three organizers specialize the fb, mb, and hb into distinct structures. The ANR continues to specify the tel as it begins to specialize, and the diencephalon, under instruction from the ZLI, has split into the prethalamus (pthal), which co-opts the role of the ANR by becoming a source of anti-WNTs, and the thalamus (thal). The mb is dominated by dorsal, midline expression of WNT signals (blue bar) from the MHB, which drives the formation of the optic tectum (tec), and the cerebellum (cere) in the hb. As the secondary organizers continue to specialize the brain around them, neurogenesis will shape and functionalize the presumptive brain structures.

As the AP neuraxis develops, Hedgehog signals (Hh) are expressed along the presumptive notochord, and confer ventral, midline identity to the overlying elongating neural plate. BMPs and FGFs from the ectoderm surrounding the neural plate suppress Hh signals in the lateral areas of the plate (Figure 2.3B, C; [Wilson and Houart 2004]). When the neural plate elongates and folds to become the neural tube, cell proliferation splits the closing tube into four domains along the dorsal/ventral (DV) axis. The ventral-most portion becomes the Hh-positive floor plate, the middle is composed of the basal plate and alar domain, and the dorsal-most portion becomes the roof plate, secreting BMPs, WNTs and FGFs (Figure 2.3C, right panel). Thus, signaling centers along the AP and DV neuraxes, established or initiated during gastrulation, now work to ensure three-dimensional cellular registry of the brain during neurulation and beyond.

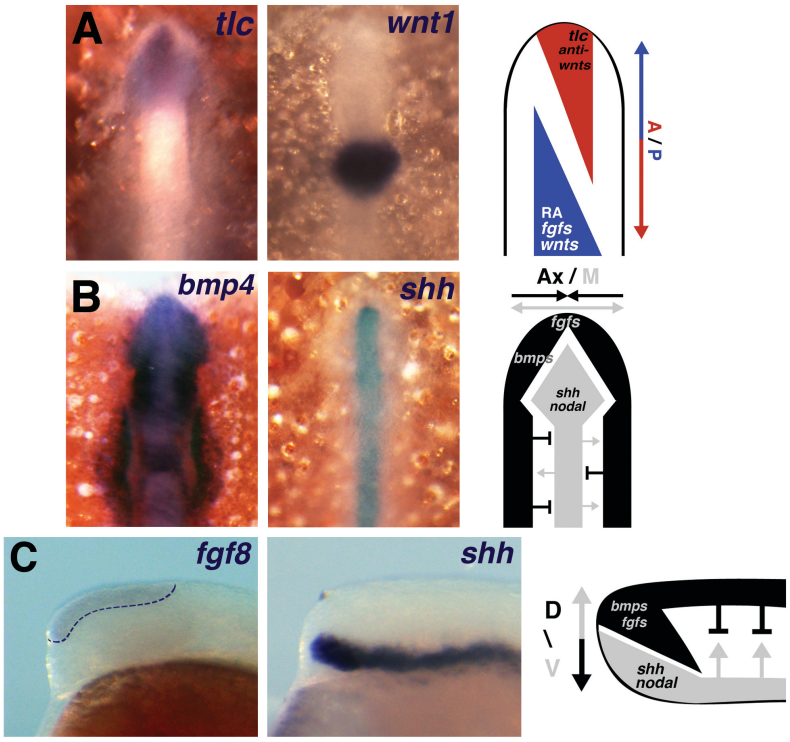

Figure 2.3 Gene Interactions Establish the Neuraxes. A The left and mid panels show the dorsal expression of *tlc* and *wnt* respectively, during late neurula in the cichlid *Metriaclima zebra*. The influence of these two genes and other factors are represented by red and blue triangles in the schematic in the right panel. The base of the triangle is the signal at its highest concentration, which decreases as it travels to the opposite end. These signals antagonize each other and establish the anterior/posterior (AP) neuraxis, represented by the dual arrowed line. **B** The interaction of signals across the axial/medial axis (AM) in the late neurula stage of the cichlid *Labeotropheus fuelleborni*. In the left panel, *bmp4* restricts *shh* expression (mid panel) to the midline of the developing neural tube. The right panel depicts this interaction as a schematic; *shh* expression works in a gradient that decreases anterior and axially, as well as from the midline of the embryo (black arrows). Conversely BMPs and FGFs work along the periphery of the embryo to restrict *shh*. This dynamic is important in the specification of the eyes, and the formation of the dorsal/ventral (DV) neuraxis. **C** The subsequent formation of the DV neuraxis is shown in *Astyanax mexicanus*. As the neural tube closes, signals along the AM axis move to establish their respective dorsal and ventral positions. Now genes like *fgf8* signal from the dorsal forebrain to counteract the ventralizing influence of *shh* from the floorplate.

As the embryo enters the neurula stage, patterning events are initiated that will subdivide the brain further, later in development. The forebrain is partitioned into three regions, the anterior telencephalon, the eye field, and the posterior diencephalon via the actions of *foxg1*, *rx*, *six3*, and *pax6* (Stigloher *et al*. 2006; Figure 2.2B). An integration of transcription factors working along both

AP and DV neuraxes drives the formation of another signaling boundary and secondary organizer, the *shh*-positive zona limitans intrathalamica (ZLI). Along the AP neuraxis, *fezf2* and *arx* on the anterior, *otx2* and WNT-activated *irx1* on the posterior side form a narrow 'lane' in the alar domain, which allows *shh* to progress dorsally from the floor plate (Scholpp *et al*. 2007; Rodriguez-Seguel *et al*. 2009). The tandem organizing activity between the newly formed ZLI and the MHB will differentiate the diencephalon from the midbrain (Echevarria *et al*. 2003).

By the end of neurula, the major divisions of the brain, as well as the eyes, have been specified (Figure 2.3C). The notochord and spinal cord develop and mesoderm-derived muscle precursors called somites begin to form along the AP axis. It is this process, somitogenesis, that marks the next major developmental stage. The ZLI becomes a discrete anatomical boundary, and is also called the Mid-Diencephalic Organizer (MDO) due to its activity in the posterior forebrain (Scholpp *et al.* 2007; Scholpp and Lumsden 2010). The ZLI, via Hh signals, specifies the thalamus and prethalamus from the diencephalon, using transcription factors *irx1* and *dlx2* respectively (Kiecker and Lumsden 2005; Vieira *et al*. 2005, Scholpp *et al*. 2006). *otx2* and *meis2* specify the tectum from the midbrain (Agoston and Schulte 2009), and WNT + FGF signals from the MHB split the cerebellum from other hindbrain-derived structures (Liu and Joyner 2001, Canning *et al*. 2007).

During somitogenesis, the secondary organizers compartmentalize the presumptive brain, and each compartment organizes into structures (Figure

2.2C). Each of these structures in turn can initiate signaling centers with downstream transcription factors. Often these 'tertiary' organizers represent familiar signals working in a new developmental context. A well-known example of the phenomena is the partitioning of the telencephalon. The telencephalon splits into a dorsal pallium and ventral subpallium mediated by dorsal WNT and ventral Hh signals, integrated by *foxg1* (Danesin *et al*. 2009); these WNT versus Hh signals begin the process during neurula. The split is reinforced by the formation of the pallial-subpallial boundary (PSB), which forms at the interface of dorsal *tbr1, emx* and *pax6* plus ventral *nkx2.1* and *dlx2* respectively (Puelles *et al*. 2000). The subpallium continues development under the influence of Hh signal from the ventral telencephalon and FGF signals from the ANR, and transcription factors like *lhx* and *foxg1* specify the olfactory primordia and basal ganglia (Menuet *et al*. 2007, Manuel *et al.* 2010). The pallium forms the cortex, which develops along the AP and DV neuraxes under a 3-D matrix of signals from the ANR and roof plate (WNTs, BMPs, FGFs) and downstream transcription factors (*pax6, emx3, sp8*) (Ohkubo *et al.* 2002, O'Leary and Sahara 2008).

Thus, somitogenesis marks (i) a further decline in the ability of signals to act globally across the major neuraxes and (ii) greater modularity of brain structures, achieved by redeploying the function of the same upstream genes in new downstream milieus. As somitogenesis proceeds, the first stage of neurogenesis occurs; rapid proliferation of the presumptive brain cause it to grow at a much faster rate than the rest of the central nervous system, causing encephalization. The ultimate size of the entire brain is influenced by the rate of

proliferation at this point, modulated by dorsal WNT and ventral Hh signals (Altaba *et al.* 2002, Fuccillo *et al.* 2006, Joksimovic *et al.* 2009*,* Alvarez-Medina *et al.* 2009). Boundaries and secondary organizers, under the control of WNT and Hh (and also FGFs and BMPs), can pinpoint proliferation specifically within compartments, brain structures, or areas within structures.

The main organizing principles to take from this section are as follows. Primary AP and DV neuraxes important to brain regionalization are initiated and/or established in gastrula to early neurula embryos. Familiar signaling molecules (BMP, WNT, FGF, Hh) act to polarize the neural plate and neural tube, similar to other organ systems like mandibles and limbs. Graded signals integrate the function of transcription factors whose primary role is to specify and provide identity to brain compartments, separated by signaling boundaries. Boundaries act to subdivide the brain into regions. Signals and transcription factors are used iteratively from stage to stage and often across the neuraxes. WNT molecules are a case in point; these function first in gastrula as one of the earliest signals of posterior commitment along the AP axis, then later as dorsal signals to split the telencephalon at neurula, and later still within compartments to facilitate neural precursor proliferation. It should be clear at this point that there is ample opportunity for evolutionary diversification along these early neuraxes – modification in the timing, position and/or activity of signaling centers may greatly impact brain development across species.

2.4 Fish as New Models of Brain Evolutionary Development

Traditionally, mammals were the primary group used in studies of vertebrate brain evolution, but recently other lineages have been incorporated, to discover and refine general evolutionary trends (Charvet *et al.* 2009, Yopak *et al*. 2010). Fishes comprise the largest vertebrate radiation, exhibit a large amount of morphological variety, and inhabit almost every aquatic habitat on Earth. Fish brains are diverse in size, architecture, and structure; variation in fish brains tends to track trophic ecology, the physical environment and social organization (Kotrschal 1998, Ito e*t al*. 2007, Streelman *et al.* 2007, Shumway *et al.* 2008, Gonzalez-Voyer *et al.* 2009a). There are several examples among fishes of nearly genetically identical populations or species exhibiting distinct ecotypes, such as the limnetic versus benthic forms in three-spined stickleback, *Gasterosteus aculeatus*, and the surface versus eyeless cave-dwelling *Astyanax mexicanus* (Jeffery 2009, Schluter 2010). Also, rapid evolutionary radiations like the cichlids of East Africa provide a wide variety of ecotypes against a similarly shared genetic background. This makes fishes an attractive system in which to study the evolution and development of the brain in natural populations, across the entirety of ontogeny, including the earliest patterning events. In particular, early development events can be compared across these closely related populations and species because embryos of distinct ecotypes exhibit similar approximate size and ontogenetic tempo.

The Mexican cavefish, *Astyanax mexicanus*, notable for intraspecific eye loss, has been used to understand evolution of the patterning networks that give rise the most anterior portion of the presumptive brain (Rétaux *et al.* 2008). The anterior-most portion of the embryonic brain consists of a combined telencephalon plus eye field (Figure 2.3B), with a ventrally located hypothalamus. The difference between the eyed surface fish and eyeless cavefish is the expansion of *shh* expression, in cavefish, along the embryonic ventral midline. Expanded Hh signaling disrupts the actions of *pax6* and *rx* transcription factors in eye development and results in eye degeneration (Yamamoto *et al*. 2004). Later in ontogeny, Hh regulates neurogenesis in the retina, which can also affect the size and functionality of the eye (Neumann and Nuesslein-Volhard 2000). In addition to these dramatic effects on the eye, the expanded redeployment of *shh* leads to (i) an enlarged hypothalamus, ventral telencephalon (the subpallium), and olfactory anlage, as well as (ii) increased neurogenic proliferation in these structures (Menuet *et al.* 2007). Such integrated changes in brain and eye morphology between eyed and eyeless forms are likely a by-product of the iterative nature of brain development (above), as downstream patterning and neurogenic networks build upon initial differences in *shh* expression from the floorplate. These early changes in *shh* along the DV neuraxis effectively shift the output of the cascading forebrain patterning network from a visually-oriented outcome in surface fish to one more suited for olfaction, important in the cavefish habitat (Figure 2.4B, D).

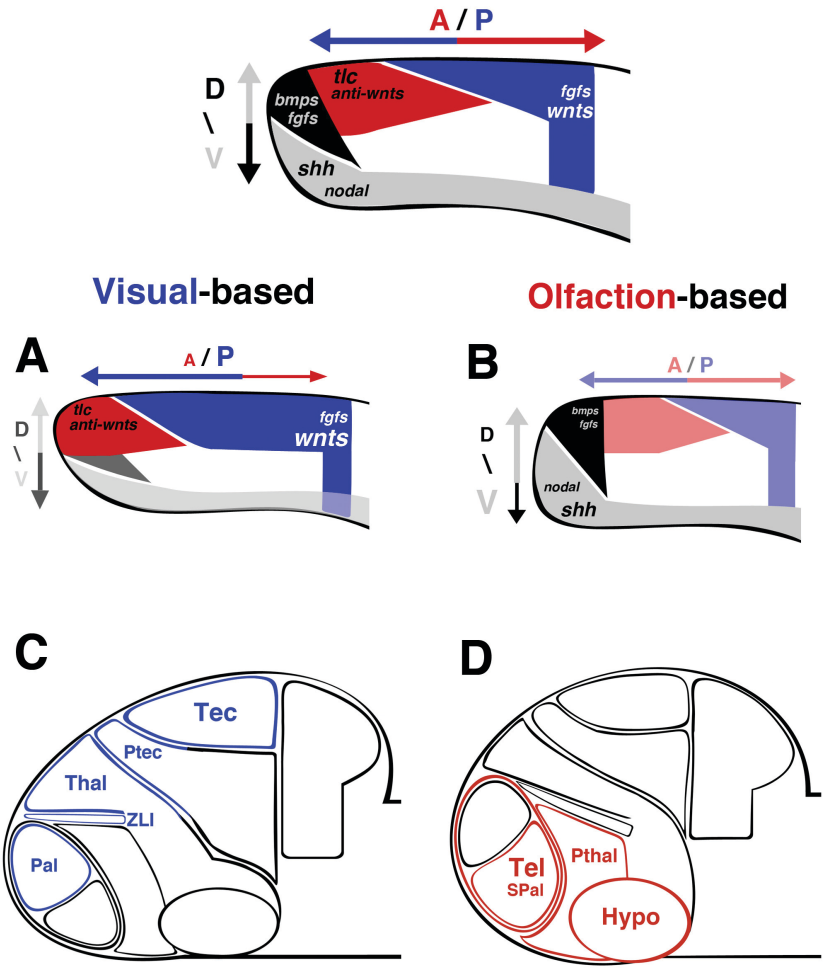

Figure 2.4 Early Changes Along the Neuraxis Modify the Layout of the Brain. The schematic at top represents the action of signaling gene gradients across the AP and DV neuraxes of the anterior neural tube in a generalized vertebrate. The neuraxes are represented by dual arrowed lines and each arrow is the direction of signal; red/blue for the AP neuraxis, and black/gray for the DV. The AP neuraxis is defined by the ANR (in red) and the MHB (blue) and the DV neuraxis is a gradient originating from the floorplate (in gray) and antagonized by signal from the roofplate (black). The major signaling genes that act along these gradients are listed on each triangle. The AP and DV neuraxes do not work independently; gene signals often work in multiple axes, thus the neuraxes are highly integrated. **A** The expansion of posterior signal via WNTs and a decrease in anterior signal defines a visual-based brain, as demonstrated in the utaka cichlids. Due to the integrated nature of neuraxes, this also changes the deployment of signals along the DV neuraxis, affecting a large portion of the presumptive brain. **B** An increase of Hh signal along the DV axis, which has been described in mbuna cichlids and cavefish, would indirectly affect signals working across the AP neuraxis. This shifts the output of patterning networks to an olfaction-based brain. **C** The actions of WNT signals after initial expansion in A can result in development and growth of the structures involved in the visual circuit (tectum – tec, pretectum – ptec, and thalamus – thal) and processing (pallium – pal), as well as a shift in the ZLI secondary organizer. **D** Stronger influence of the Hh signal in B can expand ventral structures of the brain (hypothalamus – hypo, prethalamus – pthal), as well as structures which integrate across both the AP and DV neuraxes like the tel and its downstream compartment (subpallium – spal).

The cichlids of East Africa comprise a second group of fishes where the size and shape of adult brain structures vary according to habitat, diet (van Staaden *et al.* 1994, Huber *et al.* 1997, Pollen *et al.* 2007), and/or social behavior (Burmeister 2007, Gonzalez-Voyer *et al*. 2009b). Within Lake Malawi, members of the rock-dwelling (locally called “mbuna”) versus sand-dwelling (locally, “utaka”) evolutionary lineages share similar genomes (Loh *et al*. 2008) but exhibit distinct brains and behaviors (van Staaden *et al.* 1994, Huber *et al*. 1997). Mbuna are strongly territorial (Parnell and Streelman 2011); they breed and feed at high density in complex 3D habitats. Most mbuna eat algae from the substratum. Mbuna brains have elaborated the anterior-most compartment, the telencephalon – in particular, the subpallium – and the olfactory bulb. Conversely, utaka are less site-specific, living over vast expanses of sand. Many utaka species capture small prey using acute vision; in general, their brains are elaborated for posterior structures like the optic tectum, the thalamus and the eye field.

The difference between the two lineages in brain regionalization is, in part, driven by the placement of an important signaling boundary and secondary organizer, the ZLI (Sylvester *et al.* 2010). This boundary divides the brain along the AP neuraxis and secretes Hh signal (Kiecker and Lumsden 2005, Vieira *et al*. 2005, Scholpp *et al.* 2006). The relative position of the ZLI in mbuna versus utaka is established by anterior and posterior transcription factors, which in turn are initiated via differential signals from the ANR versus MHB (Figure 2.4). Mbuna exhibit effectively stronger signaling from the ANR and elaborate anterior

structures while utaka exhibit effectively stronger WNT signals from the MHB and elaborate posterior, visually-oriented structures (Figure 2.4, (Sylvester *et al*. 2010)).

2.5 Evolutionary Divergence Along the Developing Neuraxes

Brains develop from an undifferentiated plane of cells into a highly complex, compartmentalized, 3-D organ. As this process unfolds, molecular signals establish the major brain axes and initial boundaries to subsequent signaling. Boundaries delineate compartments within which independent structures develop under the direction of transcription factors. Because brain development is a process of increasing differentiation and compartmentalization, the impact of evolutionary variation in patterning depends upon the timing of change.

Early differences in the spatial extent or strength of signaling molecules have the potential to affect large regions of the presumptive brain, because these early signals are relatively unimpeded by boundaries and because the stages of brain development are strongly contingent upon preceding events. The expansion of ventral midline Hh signal in cavefish is a case in point, which in addition to eye loss and widespread brain changes, may also lead to wider jaws with more teeth and tastebuds (Yamamoto *et al.* 2009). A second example is the WNT signal acting along the early AP neuraxis. The WNT gradient spans regions of the brain that will ultimately form the mesencephalon (midbrain), the dorsal

thalamus, the eye field and the pallium (dorsal portion of the telencephalon). The function of the mesencephalon is to receive inputs from the eyes; it then passes information to the dorsal thalamus, which relays information to the pallium for processing (Figure 2.4C) (Perez-Perez *et al.* 2003, Broglio *et al.* 2005). WNT signals function along the AP neuraxis throughout ontogeny to specify these structures, regulate their growth by controlling proliferation during neurogenesis, and promote neural connections once these structures are functional (Panhuysen *et al.* 2004, Joksimovic *et al.* 2009). In Malawi cichlids, WNT signals are expanded along the AP neuraxis in utaka relative to mbuna as early as gastrula stage, and ultimately utaka develop a larger mesencephalon, thalamus, eye field and pallium (Sylvester *et al*. 2010, Sylvester and Streelman, in preparation).

Once the brain is split into compartments by boundaries like the MHB and the ZLI, changes in patterning along the neuraxes will have more localized affects. For example, once the ZLI has formed, signaling from the ANR no longer impacts WNT signaling in the presumptive thalamus and midbrain (Sylvester *et al.* 2010).

The timing of early patterning signals as they act along a neuraxis may be crucial as well. In fact, species or ecotypes may not differ in the magnitude of a signal, but rather in the relative timing of that signal with respect to a neighboring boundary. For example, in surface-dwelling *Astyanax*, *fgf8* is expressed at 12 hpf, along the DV neuraxis (Figure 2.3C). By contrast, in the cave-dwelling form, *fgf8* expression is slightly earlier, and in turn maintains *shh* expansion in the most

rostral-ventral part of the neuraxis (Pottin *et al*. 2011). In utaka cichlids, *wnt1* expands rostrally from the MHB earlier (Sylvester *et al.* 2010) and specifies more tissue as 'posterior' prior to the establishment of the ZLI signaling boundary. Generally, due to the temporal nature of specificity along a neuraxis, if a signal is released before a boundary develops to contain it, new populations of cells can be exposed and may switch fate in response. Timing remains important even as the brain is specified and neurogenesis begins, as described by the well-known 'late equal large' model. Indeed, due to the iterative and sustained function of patterning signals (Altaba *et al.* 2002, Fuccillo *et al*. 2006, Alvarez-Medina *et al.* 2009), initial heterochrony along a neuraxis can ultimately influence the timing of neurogenesis in many related structures. For example, the same brain components involved in the olfactory circuit have the secondary action of modulating emotional and spatial memory, and the processing of sexual and social cues via the integration of the subpallium, prethalamus, and hypothalamus (Broglio *et al.* 2005, Burmeister 2007, Gonzalez-Voyer *et al*. 2009b). Therefore, the combination of 'early equals large' with 'late equals large' could facilitate the expansion of all structures involved in a particular sensory circuit.

2.6 Conclusion

Major theories of brain evolution – developmental constraint and mosaic evolution – are typically explained mechanistically by lineage-specific and/or brain structure-specific neurogenesis. In this article, we explore a contributing

role for the earliest neural patterning events to brain diversification. Early patterning differences across species or ecotypes, during the establishment of DV and AP neuraxes, can result in changes to many structures, in concert. If evolutionary change occurs later, as boundaries break connectivity among structures in the neuraxis, a mosaic pattern may result. The ability to tease apart early and subtle differences in gene expression along the neuraxes is possible in closely related fish ecotypes because they share similar genomes, developmental rates and embryo sizes. However, evolutionarily significant early patterning differences are not limited to fishes. Recent comparative studies in birds have shown that the entire brain grows larger in chicken versus quail due to a pre-neurogenenic change in precursor cell cycle rate (Charvet *et al*. 2010a). Furthermore, there is targeted expansion of the telencephalon relative to the tectum, evident prior to the onset of neurogenesis, in parakeets versus quail (Charvet *et al.* 2008, McGowan *et al*. 2010). The link between delayed neurogenesis and size of the telencephalon in mammals could be a natural consequence of differences among patterning genes along the neuraxes of the presumptive forebrain, akin to the role of WNTs on their downstream effector β-catenin and the subsequent expansion of the cerebral cortex in mutant mice (Chenn and Walsh 2002). Future study of brain evolutionary development should incorporate the entirety of brain ontogeny, from the earliest patterning events, through neuronal differentiation, to adult remodeling via neural stem cells (Grandel *et al*. 2006). Evolution has likely acted upon each of these stages of brain development to shape neural function and behavior.

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CHAPTER 3

BRAIN DIVERSITY EVOLVES VIA DIFFERENCES IN PATTERNING[2]

3.1 Abstract

Differences in brain region size among species are thought to arise late in development via adaptive control over neurogenesis, as cells of previously patterned compartments proliferate, die and/or differentiate into neurons. Here, we investigate comparative brain development in ecologically distinct cichlid fishes from Lake Malawi and demonstrate that brains vary among recently evolved lineages because of early patterning. Divergence among rock- and sand-dwellers in the relative size of the telencephalon *vs*. thalamus is correlated with gene expression variation in a regulatory circuit (composed of *six3*, *fezf2*, *shh*, *irx1b*, *wnt1*) known from model organisms to specify anterior-posterior (AP) brain polarity and position the *shh*-positive signaling boundary zona limitans intrathalamica (ZLI) in the forebrain. To confirm that changes in this co-expression network are sufficient to produce the differences we observe, we manipulated WNT signaling *in vivo* by treating rock-dwelling cichlid embryos with temporally precise doses of LiCl. Chemically treated rock-dwellers develop gene expression patterns, ZLIs and forebrains distinct from controls and untreated conspecifics, but strongly resembling those of sand-dwellers. Notably, endemic

[2] Sylvester JB, Rich CA, Loh, Y-HE, van Staaden MJ, Fraser GJ, Streelman JT (2010) Brain diversity evolves via differences in patterning. *PNAS.* 107:9718-9723

Malawi rock- and sand-dwelling lineages are alternately fixed for a single nucleotide polymorphism in *irx1b*, a mediator of WNT signaling required for proper thalamus and ZLI. Together, these natural experiments in neuroanatomy, development and genomics suggest that evolutionary changes in AP patterning establish ecologically relevant differences in the elaboration of cichlid forebrain compartments. Generally, variation in developmental patterning might lay the foundations upon which neurogenesis erects diverse brain architectures.

3.2 Introduction

The brain is arguably the best-studied vertebrate organ and it has played an important role in the evolution of our own species. Modifications of brain structure are responsible for novel behaviors that galvanized evolutionary radiation of the major vertebrate groups (Striedter 2005). Following decades of research in model organisms, we know a great deal about how the process of development makes a brain (Kiecker and Lumsden 2005). We know much less about evolutionary mechanisms of brain diversification.

The brain develops under the iterative influence of antagonistic anterior and posterior signaling molecules, inductive and repressive transcription factors that receive those signals, and lineage restriction boundaries that define compartments (Kiecker and Lumsden 2005, Wilson *et al.* 2004). Just after gastrulation, the initial anterior-posterior (AP) polarity of the brain is established by a tug-of-war between posteriorizing signals (e.g., *wnt1*) secreted from the

midbrain-hindbrain boundary (MHB) and WNT antagonists (e.g., *six3*, *tlc*) expressed from the anterior neural ridge (ANR). The MHB develops to demarcate the hindbrain from the fore- plus midbrain (Figure A1). With the subsequent formation of the diencephalon-midbrain boundary and the zona limitans intrathalamica (ZLI), the forebrain and midbrain begin to follow separate paths of development.

These initial boundaries and signaling centers (viz., ANR, MHB, ZLI) continue to direct additional patterning and morphogenesis within the three major brain regions. For example, the forebrain differentiates into rostral (e.g., telencephalon, hypothalamus) and caudal (e.g., thalamus) domains, mediated in part by the ZLI (Scholpp *et al.* 2006). As brain compartments are patterned, proliferating cells within each compartment undergo neurogenesis, maturing and differentiating into functional neurons. Because brain patterning demarcates one region from another, specific compartments may initiate, prolong and/or terminate neurogenesis independently (Streidter 2005, Finlay *et al.* 2001, Takahashi *et al.* 1996). Given the continuum of patterning and neurogenesis in brain development, vertebrate lineages might evolve brain diversity by (i) varying the strength or timing of signals from the ANR and/or MHB, (ii) shifting the position of early patterning boundaries, (iii) altering the timing, rate or extent of neurogenesis, or (iv) some combination thereof. Expectations from the field of evolutionary developmental biology suggest a focus on early patterning events since such differences prefigure the diversity of animal body plans (Gerhart and Kirschner 1997), jaws (Abzhanov *et al.* 2004, Albertson *et al.* 2005) and

dentitions (Fraser *et al.* 2008). By contrast, our understanding of the brain departs from this notion, as an extensive literature highlights the role of neurogenesis in brain diversification. Most prominently, the 'late equals large' model explains how the neocortex (i.e., telencephalon) has evolved to dominate the mammalian brain and how individual lineages (e.g., primates) have further elaborated this region by tipping the balance between neural cell proliferation, differentiation and apoptosis (Finlay *et al.* 2001, Takahashi *et al.* 1996, Finlay *et al.* 1995, Chenn and Walsh 2002). Addressing the genetic and developmental mechanisms of brain diversification in nature has been difficult, however, because few systems offer the necessary combination of a wide range of brain phenotypes and tractable experimentation with embryos, against a background of genomic and developmental similarity.

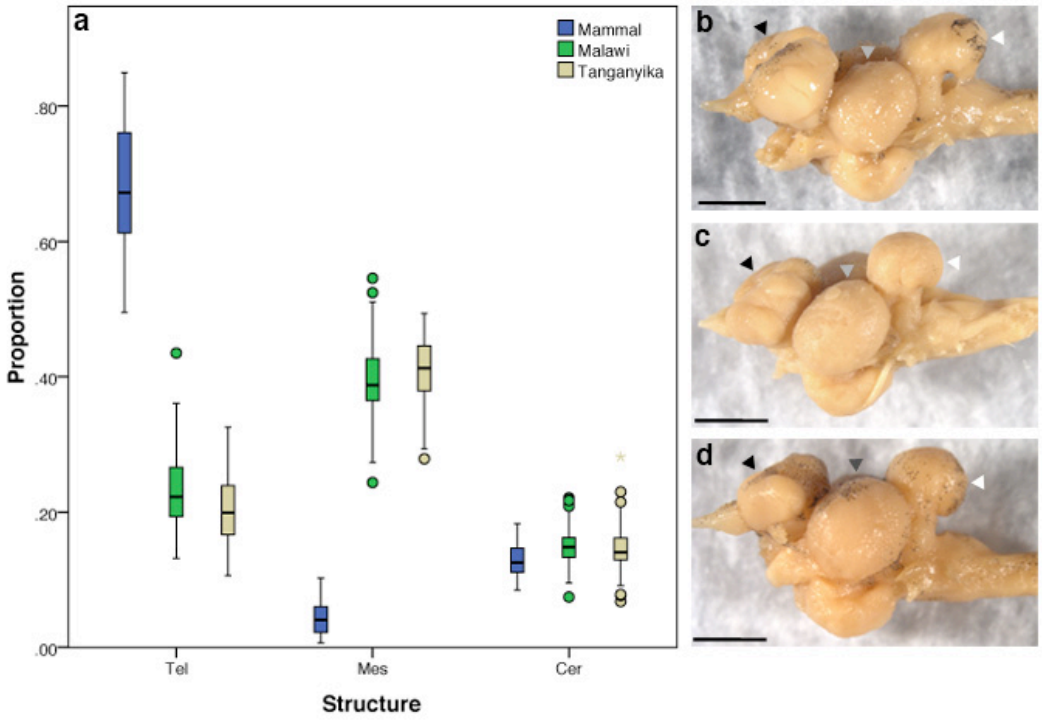

Figure 3.1. Cichlid brains are diverse. a, Cerebrotype box plots of mammals (blue), Lake Malawi cichlids (green), and Lake Tanganyikan cichlids (tan), grouped by brain proportions of the telencephalon (forebrain), mesencephalon (midbrain), and cerebellum (hindbrain). The heavy line in the middle of the box is the median value, the box itself is the 25 to 75% interval around the median, the bars are the 10 to 90% interval, and dots represent data points outside the 95% interval. Mammals have invested heavily in the neocortex (tel); cichlid brains are more proportional in their regional allocations. Despite an order of magnitude difference in divergence time, the range of variation in brain proportions is comparable from mammals to cichlids. Brains of rock-dwelling (mbuna) cichlids **b**, *Labeotropheus fuelleborni* (LF, algivore). **c**, *Maylandia zebra* (MZ, generalist). **d**, *Cynotilapia afra* (CA, planktivore) in lateral view, anterior is to the left. Black arrowheads, telencephala; grey arrowheads, optic tecta; white arrowheads, cerebella. Scale bars = 2 mm. Note that the major midbrain structure of fishes (optic tectum) is of uncertain homology to mesencephalic derivatives in mammals.

3.3 Results and Discussion

We used cichlid fishes from Lake Malawi to ask when and how brains develop diversity in recently evolved lineages. Cichlid adult brain variation is appreciable (Figure 3.1A) and is correlated with ecology and behavior (Huber *et*

al. 1997, Pollen *et al.* 2007, Shumway 2008). For example, algal scrapers exhibit small optic lobes and large telencephala (and olfactory bulbs, Figure 3.1B), while planktivores have enlarged optic lobes (Figure 3.1D); 'sonar' hunters – species that feed by sensing vibrations – have large telencephala and cerebella. This diversity, similar to that observed across seven orders of mammals (including primates, insectivores, marsupials, cetaceans and bats (Clark *et al.* 2001)), has evolved rapidly. Hundreds of Lake Malawi cichlid species have diverged from a common ancestor in the last 500k years; their genomes are highly similar and retain ancestral polymorphism (Loh *et al.* 2008). Malawi cichlid brains are thus as different as those of long-diverged mammals (~150 million years (Kumar and Hedges 1998)), but their genomes are comparable to those of any two humans. We focused on species representing the range of ecotypes in Lake Malawi. We studied brain development of three rock-dwelling (mbuna) cichlids: *Labeotropheus fuelleborni* (obligate algal scraper, LF), *Maylandia zebra* (generalist, MZ), and *Cynotilapia afra* (planktivore, CA), as well as three sand-dwelling non-mbuna: *Copadichromis borleyi* (planktivore, CB), *Mchenga conophorus* (generalist, MC), and *Aulonocara jacobfreibergi* ('sonar' hunter, AJ). Mbuna *vs*. non-mbuna comprise distinct evolutionary groups, each containing hundreds of species, with generally contrasting lifestyles, body forms, visual systems, pigment patterns and trophic adaptations (Carleton *et al.* 2008, Hulsey *et al.* 2007, Streelman and Danley 2003).

3.3.1 Cichlid forebrains differ early in development

By stage 16 (Figure A1D), Lake Malawi cichlid forebrains have been partitioned into several compartments, visualized in the para-sagittal section with the greatest dorso-ventral extent (hereafter, 'para-sagittal section'). This is the first stage at which these regions can be reliably measured (Methods, Figure A2). We quantified the area of forebrain compartments in replicate embryos of the mbuna LF, MZ, and CA, as well as the non-mbuna CB, MC and AJ. Embryonic brains show clear divergence between rock- and sand-dwelling groups (Table 3.1; Figure A3). Rock-dwellers exhibit forebrains with relatively larger telencephala and smaller thalami, and sand-dwellers display the converse pattern. Adult rock-dwelling cichlids possess larger telencephala than other habitat specialists on average, perhaps because they spend their lives navigating complex 3D habitats and/or engaging in complex social interactions (Huber *et al.* 1997, Pollen *et al.* 2007, Shumway 2008). Thalami have been less well studied in fishes, but the vertebrate thalamus is a well-known 'relay station' integrating sensory, particularly visual, stimuli (Jones 2007). Our data demonstrate that differences in cichlid forebrains are apparent early in development, and that these differences might represent a trade-off between rostral and caudal compartments corresponding to adult structures evolved for contrasting ecological demands.

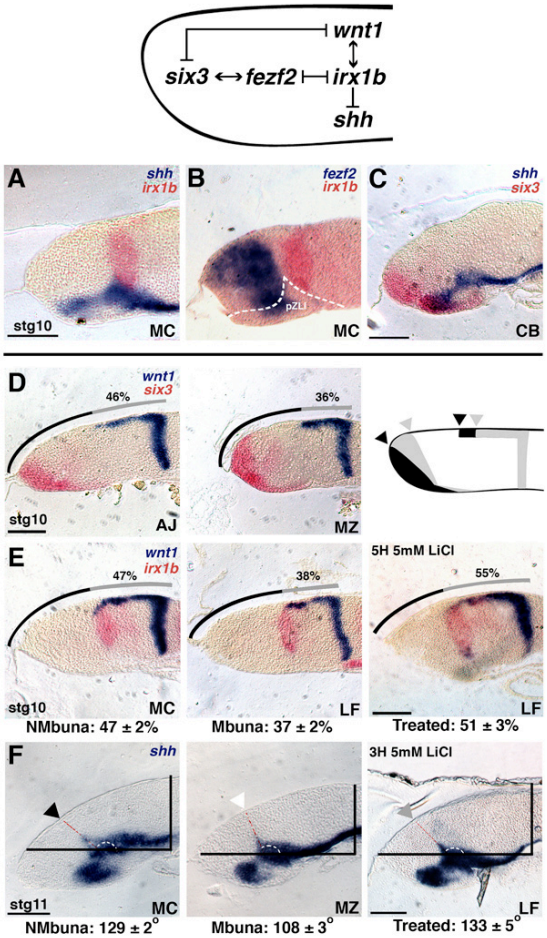

Figure 3.2. The forebrain-patterning network differs between rock- and sand-dwellers. A, double *in situ* hybridization (ISH) of genes *shh* (blue) and *irx1b* (red). **B**, double ISH of *fezf2* (blue) and *irx1b* (red); the presumptive ZLI (pZLI) is shown by the dotted white line. **C**, double ISH of genes *shh* (blue) and *six3* (red). **A** and **B** are embryos of non-mbuna *Mchenga conophorus* (MC), **C** is non-mbuna *Copadichromis borleyi* (CB); **A-C** are stage 10 embryos, scale bars represent 100 μm. **D**, from left to right, double ISH of *wnt1* (blue) and *six3* (red) in *Aulonocara jacobfreibergi* (AJ, non-mbuna), *Maylandia zebra* (MZ, mbuna), and a schematic summarizing expression differences between non-mbuna and mbuna. Arrowheads mark the relative positions of *wnt1* and *six3* expression in mbuna (gray) and non-mbuna (black), respectively. **E**, from left to right, double ISH of *wnt1* (blue) and *irx1b* (red) in MC (non-mbuna), *Labeotropheus fuelleborni* (LF, mbuna), and LF treated for 5 hours with 5mM LiCl. For all panels in **D** and **E**, the line above the embryo represents the total length of the dorsal brain anterior to the MHB and the gray portion of the bar represents the rostral extent of *wnt1* expression (the *wnt1* percentage). The measured *wnt1* percentage for each embryo, in each panel, is given. Below row **E**, from left to right, we show the average *wnt1* percentage for non-mbuna, mbuna, and LiCl-treated LF, respectively. All embryos in **D** and **E** are at stage 10; the scale bars represent 100 μm. **F**, ISH for the gene *shh* demonstrating the angle of the zona limitans intrathalamica (ZLI) at stage 11. The ZLI is marked by the black arrowhead in non-mbuna (MC), white arrowhead in mbuna (MZ), and gray arrowhead in LiCl-treated LF. The dotted red and white lines show the 'ZLI angle' (See Methods). The values below row **F** show the average ZLI angles for non-mbuna, mbuna, and LiCl-treated LF, respectively. The scale bars represent 100 μm. All images from all panels are para-sagittal sections with anterior to the left.

3.3.2 Variation in forebrain patterning prefigures morphological differences

We sought to identify the developmental signals that initiate differences in Malawi cichlid forebrains. Studies in developmental models – zebrafish, frog and mouse – set the context for our experiments (Figure 3.2, network). We focused on a gene circuit known to (i) establish anterior (e.g., telencephalon) *vs*. posterior (e.g., thalamus) fate, and (ii) position the signaling boundary ZLI within the forebrain. The transcription factor *six3* is a WNT antagonist expressed from the ANR, required for the formation of the telencephalon and ZLI (Lavado *et al.* 2008). *wnt1* is a posteriorizing signal expressed from the MHB; knockout of *Wnt1* in mouse results in a smaller thalamus, a posterior shift in the angle of the ZLI, and a larger telencephalon (Lavado *et al.* 2008). *six3* and *wnt1* direct the activity of mutually repressive transcription factors *fezf2* and *irx1*, which in turn set the AP position of the *shh*-positive ZLI (Lavado *et al.* 2008, Rodriguez-Seguel *et al.* 2009, Scholpp *et al.* 2007). Knockdown of *irx1* in zebrafish produces a posterior expansion of the ZLI at the expense of thalamus, and a shortening of the *wnt1* forebrain domain (Scholpp *et al.* 2007, Itoh *et al.* 2002). WNT signaling might induce or be mediated by Irx1 to specify posterior fate (Itoh *et al.* 2002, Gomez-Skarmeta *et al.* 2001). We hypothesized that differences between rock- and sand- embryonic forebrains are produced by temporal and/or spatial shifts in AP forebrain patterning.

Using 2-colour *in situ* hybridization (Methods), we observed expected gene expression patterns of *six3*, *wnt1*, *fezf2*, *irx1b* and *shh* in the cichlid embryonic brain. The ZLI begins to form as an initial wedge of *shh* at stage 10;

fezf2 is expressed rostral to the wedge and *irx1b* is positioned caudal to the wedge, in the presumptive thalamus (Figure 3.2A-C). The antagonists *six3* and *wnt1* are initially localized to the ANR and MHB, respectively, at the neurula stage. *wnt1* extends rostro-dorsally from the MHB as development proceeds, encompassing the presumptive midbrain and the caudal forebrain, where it is co-expressed with *irx1b*. At stage 10, *six3* and *wnt1* show contrasting distributions between mbuna and non-mbuna cichlids (Figure 3.2D). Mbuna are characterized by a shortened *wnt1* rostro-dorsal domain and more caudo-dorsal expression of *six3*; non-mbuna exhibit the opposite pattern. Despite species-specific brain shapes, the *wnt1* rostral domain marks a greater proportion of the dorsal brain anterior to the MHB in stage 10 non-mbuna CB, MC and AJ (47 ± 2%) than in mbuna LF, MZ and CA (37 ± 2%; Student's t-test, two-tailed, $t=11.78$, $p<0.0001$ [3-7 individuals of each species, N=32 embryos]; Figure 3.2D, E).

By stage 11, the ZLI is a narrowing finger of *shh* expression within the diencephalon, forming a characteristically obtuse angle with the alar domain (Figure 3.2F). We observed that the ZLI angle is greater in non-mbuna CB, MC and AJ (129° ± 2) than in mbuna LF, MZ and CA (108° ± 3, Student's t-test, two-tailed, $t=18.24$, $p<0.0001$ [2-4 individuals of each species, N=20 embryos]). The larger ZLI angle in non-mbuna, as measured at stage 11, matches the more rostro-dorsal expression of *wnt1-irx1b* and reduced *six3* domain at stage 10. We tracked the angle of the *shh*-positive ZLI from stage 11 to stage 17 (encompassing 3 days of development and the time point of forebrain compartment measurements, Table 3.1) in replicate embryos of the three mbuna

vs. non-mbuna species (2-5 embryos per species, per stage; N=120 embryos). In both mbuna and non-mbuna, the angle of the ZLI increases as thalamic and tectal structures grow and proliferate, yet non-mbuna maintain a greater ZLI angle throughout (Figure 3.3, Figure A4).

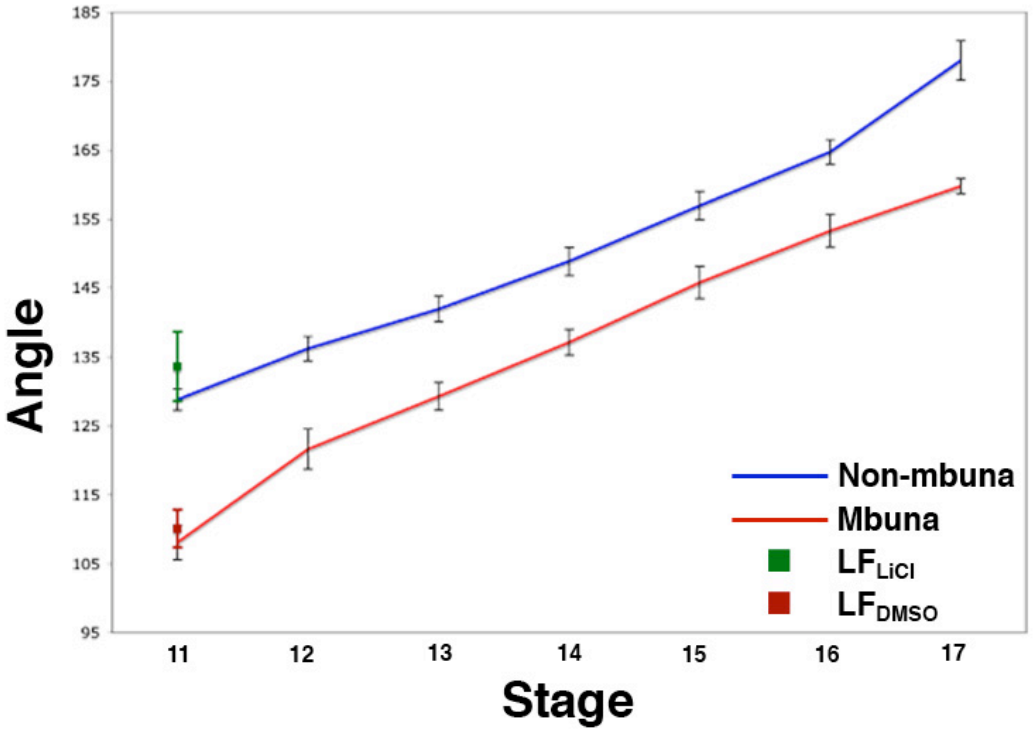

Figure 3.3. The angle of the signaling boundary ZLI impacts brain regionalization during neurogenesis and growth. The graph presents the average ZLI angle (± 1 SD, 2-5 specimens per species per stage, N=120) over seven ontogenetic stages for non-mbuna CB, MC and AJ (blue) and mbuna LF, MZ, and CA (red); see also Figure S4. The green and red data points at stage 11 represent LiCl-treated and DMSO control LF embryos, respectively.

3.3.3 Manipulation of WNT signaling mimics natural variation among cichlid forebrains

The brain and gene expression phenotypes we observed to differentiate mbuna (reduced rostro-dorsal extent of *wnt1-irx1b*, more acute ZLI angle, smaller thalamus, larger telencephalon) from non-mbuna cichlids (greater rostro-dorsal extent of *wnt1-irx1b*, more obtuse ZLI angle, larger thalamus, smaller telencephalon; Table 3.1, Figure 3.2) partially phenocopy zebrafish *irx1* knockdown *vs*. control embryos (Lavado *et al.* 2008) and *Wnt1* null *vs*. control mice (Itoh *et al.* 2002). Thus, we manipulated WNT signaling *in vivo* by treating cichlid embryos with non-lethal doses of the chemical agonist LiCl (Methods). This approach does not allow the genetic specificity of other methods such as morpholinos, but does provide the temporal precision critical to our experiments. We bathed stage 9 embryos (during which *wnt1* expression 'moves' rostro-dorsally from the MHB; Figure A1) of the mbuna LF in a 5mM solution of LiCl or a vehicle control (DMSO), for 3 or 5 hours. We then washed the embryos, returned them to fish water, and cultured them until sacrifice (i) at stage 10 to measure the rostro-dorsal extent of the *wnt1-irx1b* expression domain, (ii) stage 11 to measure the angle of the *shh*-positive ZLI, or (iii) stage 16 to measure the relative area of forebrain compartments. We predicted that up-regulation of WNT signaling would transform the treated mbuna brains of LF to resemble those of non-mbuna.

At stage 10, LiCl-treated LF (N=6; 3 and 5 hour treatments combined) exhibited *wnt1-irx1b* expression domains that covered a greater proportion of the

dorsal brain anterior to the MHB (51 ± 3%) than did DMSO controls (N=4, 37 ± 1%; Student's t-test, two-tailed, t=9.05, p<0.0001; Figure 3.2E, right panel). At stage 11, LiCl-treated LF (N=4; 3 and 5 hour treatments combined) exhibited greater ZLI angles (133 ± 5°) than did DMSO controls (N=2, 110 ± 3°; Student's t-test, two-tailed, t=5.96, p=0.004; Figure 3.2F, right panel). Finally, at stage 16, LiCl-treated LF (N=5; 3 and 5 hour treatments combined) exhibited smaller telencephala (27.7 ± 1.3%) and larger thalami (24.7 ± 0.9%) than did DMSO controls (N=3; 31.7 ± 0.6% and 20.6 ± 0.6% for telencephala and thalami, respectively; Table 1). The areas of prethalami and hypothalami did not differ between treatment and control embryos (Table 3.1). Our measurements for LF bathed in DMSO (controls) fell within the statistical distribution for untreated mbuna, and the LiCl-treated LF embryos possessed *wnt1-irx1b* gene expression patterns, ZLIs and forebrains that strongly resembled those of non-mbuna (Table 3.1; Figures 3.2 and 3.3). These data demonstrate that manipulation of WNT signaling during early embryogenesis is sufficient to produce distinct cichlid forebrains, which nearly exactly mimic the natural developmental differences between rock- and sand-dwellers.

Table 3.1 Composition of the cichlid embryonic forebrain at stage 16. Mbuna (LF, MZ and CA) possess larger telencephala and smaller thalami than non-mbuna (CB, MC and AJ). Control (DMSO) and treated (LiCl) LF embryos exhibit similar proportions to mbuna and non-mbuna cichlids, respectively. The area of each compartment is expressed as a percentage (± 1 SD) of total forebrain area.

	% Tel	% Thal	% Prethal	% Hypothal
MZ (N=5)	35.5 ± 0.8	18.1 ± 0.7	15.3 ± 1.4	31.1 ± 1.5
CA (N=4)	33.9 ± 2.2	19.4 ± 0.4	16.2 ± 0.5	30.5 ± 2.5
LF (N=5)	32.8 ± 0.5	20.9 ± 0.5	15.3 ± 2.1	30.9 ± 2.0
LF_{DMSO} (N=3)	31.7 ± 0.6	20.6 ± 0.6	16.1 ± 1.2	31.6 ± 0.6
LF_{LiCl} (N=5)	27.7 ± 1.3	24.7 ± 0.9	15.7 ± 0.3	31.9 ± 0.8
MC (N=5)	28.2 ± 0.7	24.9 ± 0.9	15.6 ± 1.3	30.8 ± 1.2
CB (N=5)	28.0 ± 0.8	27.7 ± 0.4	15.4 ± 1.5	28.8 ± 1.3
AJ (N=4)	28.4 ± 0.4	26.0 ± 0.7	15.5 ± 0.8	30.1 ± 0.8

3.3.4 A SNP in irx1b is alternately fixed between rock- and sand-dwellers

We wanted to identify genetic differences between mbuna and non-mbuna that might contribute to variation in gene expression and brain phenotypes. We did not employ the typical approach to map quantitative trait loci responsible for phenotypic variance (Albertson *et al.* 2005, Streelman and Albertson 2006) because we could not effectively cross chosen rock- with sand-dweller individuals. Therefore, we explored an alternative strategy. Because mbuna and non-mbuna evolutionary groups diverged recently, the genomes of individuals share polymorphism across the lineage boundary (Loh *et al.* 2008). Loci that are strongly genetically differentiated between lineages are statistical outliers. For instance, only one of 96 independent single nucleotide polymorphisms (SNPs) is

alternately fixed in a large sample of Malawi mbuna *vs*. non-mbuna. The SNP is a replacement change in the 3' coding sequence of the transcription factor *irx1b*, fixed between endemic Malawi mbuna [25 species, 140 alleles] and non-mbuna [52 species, 230 alleles] (Figure A5, for methods, see Appendix A Text). This represents a signature of divergent selection against a background of shared polymorphism (Loh *et al.* 2008), and suggests that genetic variation in the *irx1b* cistron might play a role in the differentiation of Malawi cichlid forebrains. At this point, we are uncertain if this substitution itself is causative or linked (physically

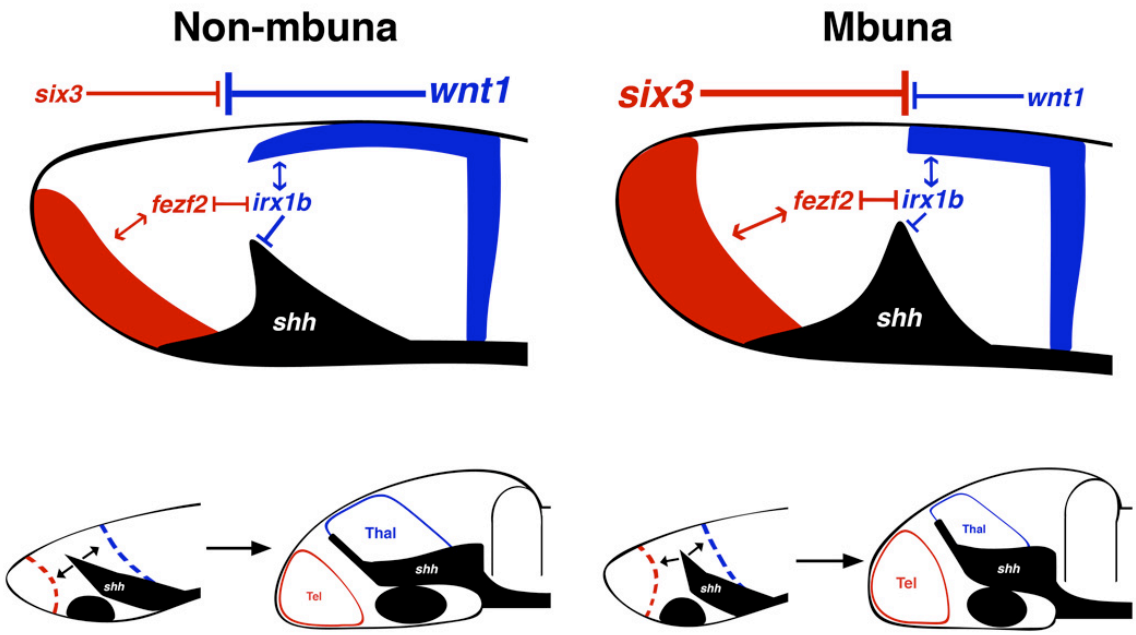

Figure 3.4. Brain diversity develops at the boundaries. A model summarizes the evolutionary developmental differences between non-mbuna and mbuna forebrains. At stage 10 (top), the allocation of forebrain structures is determined via the competing influence of posteriorizing factors from the MHB (e.g., *wnt1* shown in blue) versus WNT antagonists expressed from the ANR (e.g., *six3* in red). This in turn sets the position and angle of the presumptive ZLI (black). In non-mbuna, posterior factors dominate the forebrain, establishing a greater (more obtuse) ZLI angle relative to mbuna. This results in the differential allocation of cells to anterior *vs*. posterior forebrain compartments. During subsequent stages 11-16 (bottom panels), the initial difference in ZLI angle set during early patterning persists, with the consequence of a smaller telencephalon (tel, red) but larger thalamus (thal, blue) in non-mbuna *vs*. mbuna.

and/or epistatically) to other causal mutations, but the central position of *irx1b* in the forebrain-patterning network (Figure 3.2) implies that mbuna *vs*. non-mbuna alleles might differentially interact with *fezf2*, *wnt1* and/or *shh*. These data, coupled with the discovery that two amino acid changes between the human *vs*. chimpanzee gene *Foxp2* can drive considerable transcriptional variation in the brain (Konopka *et al.* 2009), makes cichlid *irx1b* a prime target of future study.

3.3.5 Brain diversity by patterning differences

We interpret our natural experiments in comparative neuroanatomy, development and genomics, coupled with functional information from model organisms, to indicate that evolutionary modifications in a gene circuit composed of *six3*, *fezf2*, *irx1b*, *wnt1* and *shh* establish two distinct modes of AP forebrain patterning in mbuna *vs*. non-mbuna Malawi cichlids (Figure 3.4). Non-mbuna embryonic forebrains are dominated by posterior signals (e.g., *wnt1*) and ultimately elaborate a posterior structure (thalamus) while their mbuna counterparts are under greater influence of the ANR (e.g., *six3*) and elaborate the telencephalon. Anterior and posterior signaling gradients converge on the transcription factors *fezf2* and *irx1b*, which integrate these cues to position the angle of the ZLI. This angle, once set, is important because it (i) apportions cells to anterior *vs*. posterior forebrain and (ii) represents a boundary restricting further AP cross-talk between ANR and MHB. Our data highlight early patterning variation that initiates contrasting forebrain bauplans in rock- *vs*. sand-dwelling Malawi cichlids. Species- and ecotype-specific adult brains (e.g., Figure 3.1) are likely the result of this initial difference, modified throughout ontogeny by neurogenesis. Thus, the initial angle of the signaling boundary ZLI may impact subsequent forebrain patterning and neurogenesis in ecologically distinct Malawi cichlids.

We suggest a new interpretation of how organisms evolve brain diversity: differences in the earliest signaling and patterning centers establish divergent blueprints for elaboration during growth and neurogenesis. Such comparisons

are difficult to make in mammals because deep evolutionary distances separate lineages and considerable size variation obscures brain adaptations (Finlay and Darlington 1995, Clark *et al.* 2001, Barton and Harvey 2000). Our conclusion extends those from studies of cavefishes and birds (Menuet *et al.* 2007, Striedter and Charvet 2008), and suggests that evolution might capture any point along the continuum of patterning and neurogenesis as neural systems diversify.

3.4 Materials and Methods

3.4.1 Cerebrotype Analysis

Mammalian brain data (multiple individuals of 75 species spanning seven taxonomic orders) are from the supplementary material of Clark *et al*. The Malawi data (222 individuals from 113 species) represent a combination of (i) published material (van Staaden *et al.* 1994) and (ii) new measurements made on preserved, wild-caught (from 2005), adult specimens for this study. The Tanganyika data (58 individuals, 53 species) are entirely from reference van Staaden *et al*. Volumes for the Malawi and Tanganyika measurements were generated using the ellipsoid model, $(LxWxH)\pi/6$ (van Staaden *et al.* 1994, Pollen *et al.* 2007). All cerebrotypes were calculated using the method of Clark *et al* (Clark *et al.* 2001). Box plots were generated using SPSS 16.0.

3.4.2 Embryo staging

Embryos were removed from the mouths of brooding females approximately 24 hours after identification, and, if required, maintained for further development in culture flasks at 28°C. Embryos were observed every 8-12 hours until they reached the desired stage. Stages were determined for Malawi cichlids by comparing embryonic morphology to zebrafish (Kimmel *et al.* 1995) and tilapia (Fujimura and Okada 2007) descriptions. Identification of fish brain anatomy follows Wulliman and Puelles 1999.

3.4.3 Embryonic forebrain measurements

We measured the areas of forebrain compartments in mbuna: *Labeotropheus fuelleborni* (LF), *Maylandia zebra* (MZ) and *Cynotilapia afra* (CA), and in non-mbuna: *Copadichromis borleyi* (CB), *Mchenga conophorus* (MC) and *Aulonocara jacobfreibergi* (AJ). We chose these species (LF, algal scraper; MZ, generalist; and CA, planktivore; CB, planktivore; MC, generalist; AJ, sonar hunter) to represent the range of the ecological diversity within these evolutionary lineages. Measurements were made at the first developmental stage (stage 16) where compartments are demarcated by cellular restriction boundaries and/or cellular behavior (Figure A2). All measurements were made on scaled digital images of para-sagittal sections, with ImageJ. Additionally, we used anatomical landmarks and gene expression patterns to ensure that measurements were taken from comparable serial sections (e.g., Figure A2, A3, A4). We defined the total forebrain area as the region anterior to the posterior commissure, including

the hypothalamus (Wullimann and Puelles 1999, Puelles and Rubenstein 2003). The pretectum was not included in our measurements because it could not be consistently visualized in para-sagittal section. The prethalamus included the preoptic region and the hypothalamus included the presumptive posterior tuberculum (Wullimann and Puelles 1999). To eliminate the confounding effect of oblique sections, we typically sectioned 10-20 embryos per species (often from different broods) and selected 4-5 for measurement. The area of each compartment was expressed as a percentage of total forebrain area.

3.4.4 in situ hybridization

in situ hybridization (ISH) experiments are based on published protocols (Fraser *et al.* 2008), with modification for double ISH. Gene sequences were derived from partial genome assemblies of Lake Malawi cichlids (Loh *et al.* 2008). Probes were constructed from cDNA sequences identical in the species examined; in general, Lake Malawi cichlids exhibit genetic variation comparable to that observed across laboratory strains of zebrafish (Loh *et al.* 2008). Embryos were hybridized with both fluorescein (Roche) and digoxigenin labeled RNA probes. The fluorescein was visualized first, by treating the embryos with anti-fluorescein-ap sheep antibody (Roche), then FastRed tablets (1 tablet every 2 ml of 0.1M Tris-HCl, Roche). Once the color reaction was complete, the antibody was inactivated with 0.1M Glycine-HCl (Polysciences). Embryos were then fixed briefly in 4% PFA, and the digoxigenin labeled probes were visualized as described (Fraser *et al.* 2008). All ISH experiments were performed with multiple

specimens (multiple individuals fixed at regular intervals, within single broods, then repeated at least twice with alternative broods) to fully characterize expression patterns within and across species. Embryos were embedded in gelatin and chick albumin with 2.5% gluteraldehyde. The gelatin-albumin blocks were post-fixed in 4% PFA before sectioning. Thin sections were cut at 15 μm using a Leica Microsystems VT1000 'vibratome' and imaged using a Leica Microsystems compound microscope (DM2500).

3.4.5 Measuring the rostro-dorsal extent of wnt1 expression

At stage 10, *wnt1* is expressed in the midbrain-hindbrain boundary (MHB) and also rostrally along the dorsal surface of the cichlid embryonic prosencephalon (midbrain plus forebrain). *wnt1* is a posteriorizing signal that functions across the dorsal embryonic brain (Wilson and Houart 2004, Lavado *et al.* 2008). As such, we wanted to calculate and compare – among mbuna *vs*. non-mbuna – the percentage of the dorsal prosencephalon under the influence of the *wnt1* signal. From photographs of embryos in para-sagittal section, we used Image J to measure the length of a curved line from the MHB to the rostral-most tip of the embryo, generally identified by a noticeable 'lip' demarcating dorsal from ventral (see Figure 2). This represents the total length of the dorsal prosencephalon. We next measured the rostral extent of *wnt1* expression and calculated the percentage of the dorsal prosencephalon covered by *wnt1* expression. We performed these measurements in replicate embryos of mbuna *vs*. non-mbuna species, as well as in LiCl treatment *vs*. control LF embryos.

3.4.6 Measuring the angle of the ZLI

At stage 11, *shh* expression in the ZLI forms a characteristic angle with *shh* expression in the alar domain; this angle persists during subsequent stages. The ZLI angle was measured from stage 11-17 using Image J, from photographs of para-sagittal sections. To standardize measurement across developmental time points, two guidelines were added to each image (e.g., Figure 2.2, Figure A4). The first marks the position of the vertical midbrain-hindbrain boundary (MHB) and the second is perpendicular to the first, and typically parallel to *shh* expression in the alar domain. This second line served as a consistent reference point across all stages to account for any irregularities, in section, of the *shh* alar domain. The ZLI angle was measured using the second line as the 'base' of the angle, and the position of the ZLI as the 'arm' of the angle. Multiple (2-5) embryos per species of the mbuna LF, MZ and CA, as well as non-mbuna CB, MC and AJ, were measured across the seven developmental stages (N=120 embryos).

3.4.7 Chemical treatments

A 4M lithium chloride (LiCl) stock solution was made by dissolving 640 mg of high purity LiCl (Alexis Biochemicals) into 15 mL of Dimethyl Sulfoxide (DMSO, MP Biomedicals). The LiCl stock was diluted to a final experimental concentration of 5mM (8.75 µl 4M LiCl in 7 mL of fish water). Embryos were taken from the mbuna, *Labeotropheus fuelleborni* (LF), at stage 9, around 36 to

40 hours post fertilization. Approximately 10-12 individuals were placed in separate 5mM LiCl cultures for either 3 or 5 hours, at 28°C. Additionally, 5-7 embryos were placed in 0.125% DMSO (8.75 µl DMSO in 7 mL fish water) for 3 or 5 hours, at 28°C. After treatment, embryos were washed twice with fish water, and placed in fresh fish water, in culture flasks at 28°C. Embryos were removed from culture and sacrificed at (i) stage 10 to measure the rostro-dorsal extent of the *wnt1-irx1b* expression domain, (ii) stage 11 to measure the angle of the *shh*-positive ZLI, or (iii) stage 16 to measure the relative area of forebrain compartments. The experiment was repeated twice, with two different LF broods. Treatment and control embryos were post-processed (ISH, sectioning, measurements) identically to descriptions above for untreated embryos.

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CHAPTER 4

COMPETING SIGNALS DRIVE TELENCEPHALON DIVERSITY

4.1 Abstract

The telencephalon is one of most complex and intensely studied brain structures. There are many studies that investigate the evolutionary expansion of one area of the telencephalon, the cerebral cortex, but little is known how evolution has acted on other areas. All the areas of the adult telencephalon derive from two major developmental divisions, the pallium and sub-pallium, split by the pallial-subpallial boundary (PSB). We use cichlid fishes from Lake Malawi in East Africa to demonstrate an evolutionary shift in the PSB between cichlids in a rock-dwelling lineage (mbuna) versus a sand-dwelling lineage (non-mbuna). Mbuna exhibit a dorsal shift of the PSB, and an elaboration of the subpallium, whereas non-mbuna show an opposite shift and an expansion of the pallium. Comparative and manipulative data suggest that this shift is driven by a modulation in Hedgehog (Hh) signal, part of a previously described gene circuit that establishes the position of the PSB. We describe a novel interaction between the Hh and Wingless (WNT) pathways that integrates genes working along the dorsal/ventral (DV) and anterior/posterior (AP) axes in order to set the PSB. We propose that the PSB forms at the interface of two competing pathways, mediated ventrally by Hh and *foxg1*, and dorsally by WNT and *gli3*

4.2 Introduction

The rapid expansion of the cerebral cortex is one of the key innovations in mammals, exemplified by humans. The cortex is the dorsal-most part of the telencephalon, a forebrain-derived structure that is present in all vertebrates. Whereas the underlying mechanism behind the expansion of the cortex has been explored in mice (Chenn and Walsh 2002), and the specification and function of structures within the telencephalon is known (Hebert and Fishell 2008, O'Leary and Sahara 2008), only recently have scientists begun to examine how evolution changes and refines the development of these regions in vertebrate lineages (Menuet *et al.* 2007, Charvet and Streidter 2008, Sylvester *et al.* 2010, Pottin *et al.* 2011).

All of the regions in the adult vertebrate telencephalon originate from an early developmental subdivision called the pallial-subpallial boundary (PSB), which splits the embryonic telencephalon into the dorsal pallium and ventral subpallium. The pallium is the source of excitatory neurons in the telencephalon (Yun *et al.* 2001, Cocas *et al.* 2011), and will develop into structures analogous to the mammalian cortex (Wullimann and Puelles 2004). In addition to being responsible for memory, learning, and problem solving (Broglio *et al.* 2005, Rodriguez *et al.* 2005), the pallium also interprets certain sensory signals, like vision (Sur and Rubenstein 2005). The subpallium gives rise to the inhibitory neurons (Bardet *et al.* 2010) and matures into both the basal ganglia, which

integrate the higher-thought processes of the pallium with inputs from other regions of the brain and the olfactory bulbs, which process smell (Besse *et al.* 2011, Cocas *et al.* 2011)

The pallium and subpallium are under the influence of Wingless (WNT) and Hedgehog (Hh) signals respectively (Rallu *et al.* 2002, Thiel *et al* 2002, Altaba *et al.* 2003, Machold *et al.* 2003, Backman *et al.* 2005, Bardet *et al.* 2010). These morphogens are part of separate, yet non-mutually exclusive pathways that compete for influence along the dorsal/ventral (DV) axis of the developing brain (Fuccillo *et al.* 2006, Joksimovic *et al* 2009, Ulloa and Marti 2010). The PSB forms at the interface between dorsal WNT and ventral Hh signal. A recent study by Danesin *et al.* (2009) discovered the role of *foxg1* in mediating these conflicting signals. They showed that *foxg1* is induced by *shh*, and in turn directly represses *wnt8* in the telencephalon. There is a distinct temporal order of these events; *shh* activates *foxg1* during neurulation, *foxg1* becomes self-sufficient and blocks *wnt8* with no further input from *shh* (Danesin *et al* 2009). However, if Hh signaling is disrupted during this crucial window, *foxg1* is not turned on and *wnt8* expression expands to convert the entire telencephalon into pallium. The time sensitive interplay between *shh*, *foxg1*, and *wnt8* could have important implications in establishing the position of the PSB and the resulting relative size of the pallium/subpallium

Cichlid fishes are emerging as an excellent system for studying brain diversification in vertebrate lineages. Adult cichlid brains exhibit wide variability in the size of its component structures that is linked with ecology and behavior,

but genome differences between species are comparable to any two humans, which indicates brain variation was achieved exclusive of large genetic change (Pollen *et al.* 2007, Shumway 2008, Loh *et al.* 2008, Gonzalez-Voyer *et al.* 2009, Sylvester *et al.* 2010). We investigate the Lake Malawi cichlids, which can be grouped into two lineages, loosely based on habitat. The rock-dwellers (locally and hereafter called “mbuna”) are strongly territorial; they breed and feed at high density in complex 3-D habitats. Most mbuna eat algae from the substratum. Mbuna brains have elaborated the anterior-most compartments, the telencephalon and olfactory bulbs. Sand-dwellers (locally called “utaka,” hereafter called non-mbuna) are less site-specific, living over vast expanses of sand. Most non-mbuna capture small prey using acute vision; their brains are elaborated for more posterior structures optic tecta, thalamus and eye field.

We use these lineages to show that the relative position of the PSB in the telencephalon differs between mbuna and non-mbuna; this difference is correlated to adult ecological differences. We first quantify the PSB by measuring the pallial and subpallial compartments of the telencephalon. We postulate that the PSB shift could be driven by the earlier DV interactions between *wnt8*, *foxg1*, and *shh*. We investigate this gene circuit in each lineage and document how differences in the deployment of the genes correlate to the later PSB shift. Finally, we experimentally manipulate the PSB circuit by targeting either the Hh or WNT pathway in both mbuna and non-mbuna cichlids and demonstrate that manipulation of either pathway is sufficient to change the position of the PSB.

4.3 Results

4.3.1 Proportional differences in the pallial/subpallial compartments of the telencephalon

The cichlid telencephalon is split into the dorsal pallium and the ventral subpallium by the pallial-subpallial boundary (PSB) during somitogenesis. After the establishment of the PSB (stage 13), yet before telencephalon eversion (stage 16), the pallium and subpallium can be visualized *in toto* with transverse sections and measured. When we measure the proportion of the telencephalon composed of pallium *vs*. subpallium, there is a difference in regional allocation between mbuna and non-mbuna. Mbuna embryos possess proportionally larger subpallia and smaller pallia, and non-mbuna exhibit the opposite trend (Students T-test, $t=17.218$, $p<<0.001$, Figure 4.1A; Table 4.1 shows data for 3 species of mbuna and 3 species of non-mbuna, all measured at stage 13-15). This can be nicely observed by stage 15, when the PSB can be characterized by *emx3* in the pallium and *dlx2* in the subpallium. Figure 4.1A shows a frontal section through the telencephalon and uses a 'split-screen' effect to show proportional differences between mbuna (left half-image) and non-mbuna (right half-image) – this approach works well because mbuna and non-mbuna embryos are of similar absolute size. These data suggest an important degree of variation along the DV axis between mbuna *vs*. non-mbuna species.

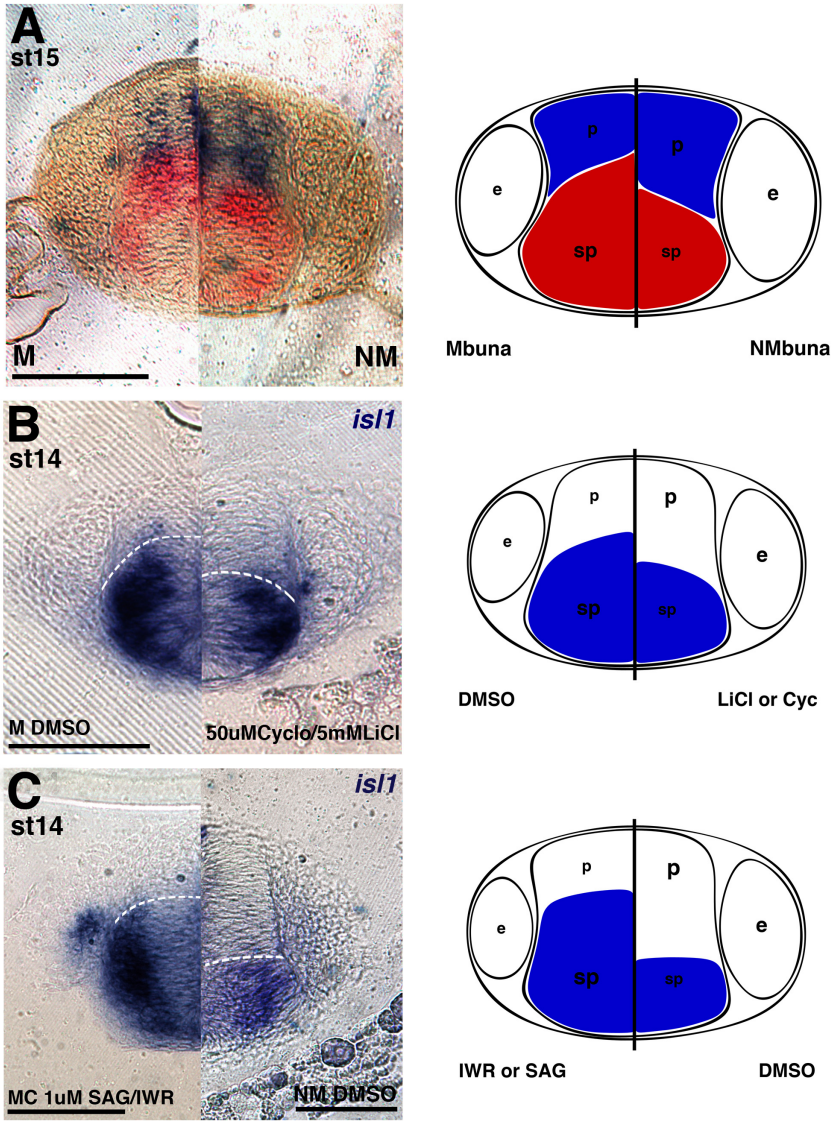

Figure 4.1. Cichlids differ in the position of the PSB. A-C, left panels are frontal sections of the telencephalon at its greatest DV extent, right panels are schematic representations of data depicted on the left. Species shown are *Maylandia zebra* for mbuna, and *Mchenga conophorus* for non-mbuna. Scale bars are 100 um. **A** is a 'split screen,' double *in situ* hybridization of *emx3* (blue) and *dlx2* (red) which visualizes the PSB in mbuna, left, and non-mbuna, right. **B** shows a ventral shift of the PSB (dotted white line) in cyclopamine or LiCl treated mbuna (right side) versus DMSO control mbuna (left side). **C** depicts a dorsal shift of PSB postion in SAG or IWR treated non-mbuna (right side) versus DMSO control non-mbuna.

4.3.2 PSB placement is established via the actions of shh, wnt8, and foxg1

In the zebrafish, DV regionalization in the telencephalon is controlled by a regulatory circuit comprised of Hh signals from the ventral floor plate and WNT signals from the dorsal roof plate, mediated by the transcription factor *foxg1* (Danesin *et al*. 2009). Specifically, *shh* induces the expression of *foxg1* in the presumptive telencephalon at neurula stage. *foxg1*, which regulates ventral telencephalic fate (Roth *et al*. 2010; Manuel *et al*. 2010), inhibits the dorsalizing effects of *wnt8* from the roof plate. We have examined the components of this regulatory circuit in Malawi cichlids.

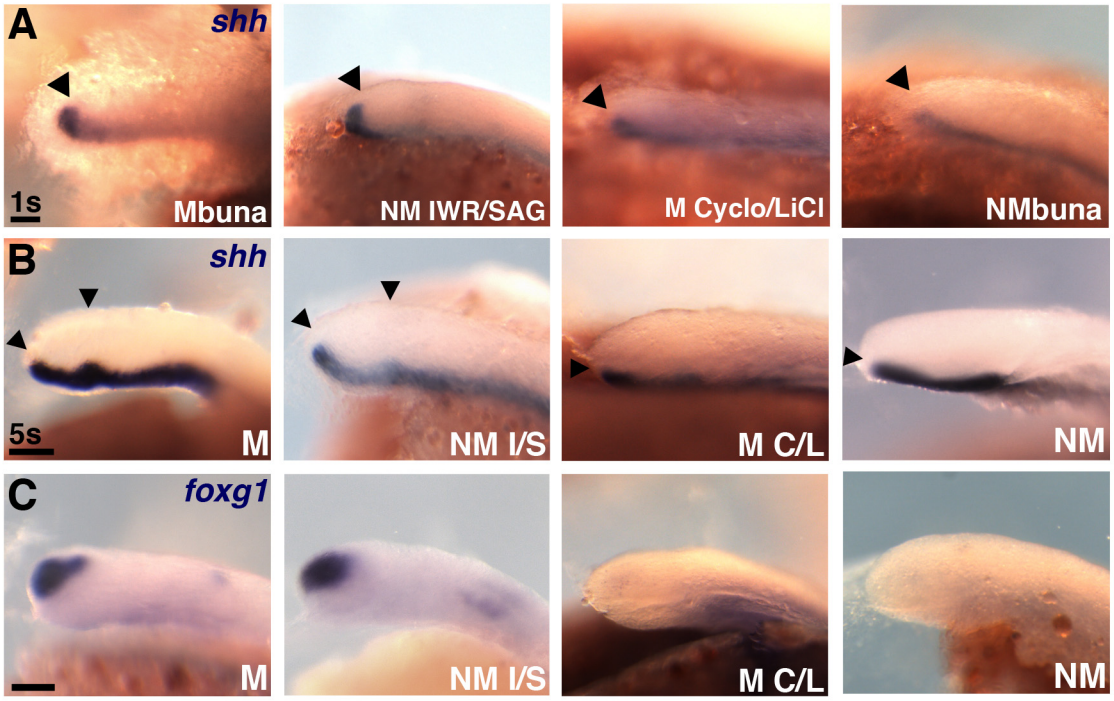

Figure 4.2. Manipulations of the Hh and WNT pathways affect the PSB gene circuit. **A-C** Images are lateral images of whole-mount embryos, anterior to the left. The panels from left to right is normal mbuna, SAG or IWR treated non-mbuna, cylopamine or LiCl treated mbuna, and normal non-mbuna. 1s in **A** and 5s in **B** refer to one somite and five somites, respectively. **C** is also five somites. Scale bars are 100 um, and apply to all 4 panels per row. **A** and **B**, arrows point to the dorsal progression of *shh* expression. Note that *shh* expression is enhanced in treated non-mbuna, but suppressed in treated mbuna. **C** depicts *foxg1* expression from normal mbuna and treated non-mbuna to a loss of *foxg1* expression in normal non-mbuna. The treated mbuna and non-mbuna demonstrate that the timing of *foxg1* induction can be experimentally altered.

The first relevant difference between cichlid lineages is that Hh signals are expressed earlier and further dorsally, in the floor plate of mbuna cichlids. In early somitogenesis (1 to 4 somites), mbuna *shh* shows stronger expression in the anterior-most section of the embryo (Figure 4.2A, left-most panel). The *shh* domain continues to be expressed more strongly from the mbuna floor plate and by 5 somites, the early and stronger *shh* expression in mbuna is correlated with *foxg1* expression in the presumptive telencephalon at 5 somites (Figure 4.2B, left-most panel). The non-mbuna *shh* domain is consistently more dorsally

restricted than mbuna, progresses dorsally more slowly, which consequently results in a delay of *foxg1* expression. Non-mbuna *foxg1* does not appear until late stage 11 (10-12 somites).

Whereas mbuna exhibit faster Hh progression along the DV axis, non-mbuna show faster WNT progression along the AP axis. As previously described with *wnt1* (Sylvester *et al.* 2010), *wnt8* also progresses more quickly out of the MHB into the presumptive midbrain and diencephalon (data not shown). Because of the actions of anti-WNTs in the anterior neural tube (Sylvester *et al* 2010), *wnt8* does not begin to move into the telencephalon until late stage 11, concomitant with the formation of the ZLI. It as this point that the heterochrony of *foxg1* comes into play; in mbuna, *foxg1* has been expressed for approximately 10 hours (time needed to develop from 5s to late stage 11), already has specified a significant portion of the anterior neural tube as telencephalon, and *wnt8* has a correlated decrease in expression in the remaining neural tissue. However in non-mbuna, *foxg1* only just begins to be expressed as *wnt8* moves into the tel. Therefore *wnt8* is able to influence a much larger proportion of the presumptive telencephalon before *foxg1* is able to block its expression. By stage 12, the telencephalon is specified with *foxg1* expression ventrally, and *wnt8* expression dorsally (Figure 4.3). The PSB will form at the interface of these two genes via downstream genes at stage 13 (e.g. *dlx2*, *isl1* in the subpallium, *pax6, emx3,* in the pallium, Figure 4.1).

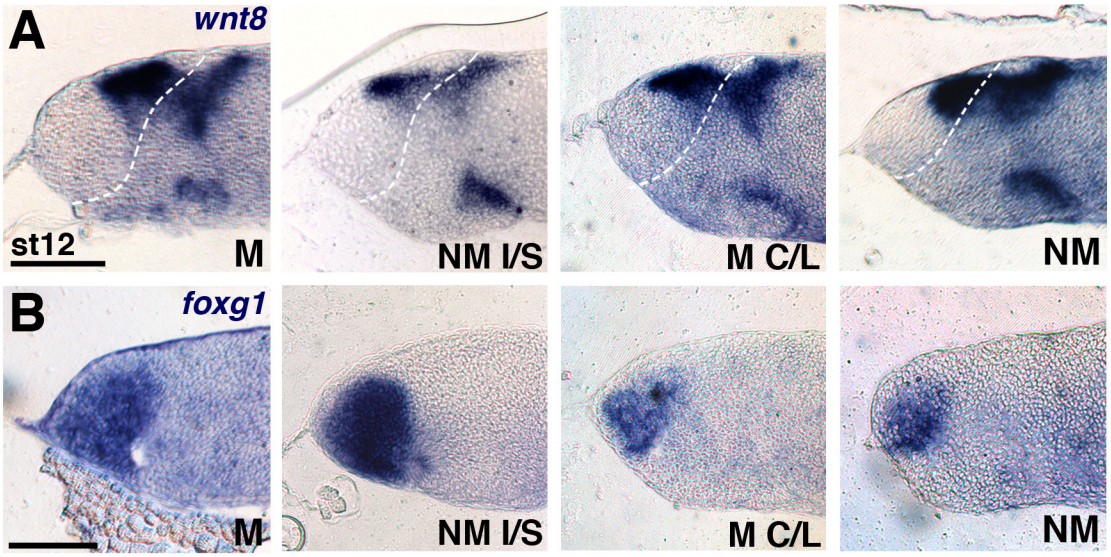

Figure 4.3. The presumptive PSB forms at the interface of *wnt8* and *foxg1*. All images are sagittal midline sections. Anterior is to the left. NM I/S refers to non-mbuna treated with either IWR or SAG, M C/L refers to mbuna treated with either cyclopamine or LiCl. Species depicted here are *Labeotropheus fuelleborni* for mbuna, *Mchenga conophorus* for non-mbuna. Scale bars are 100 um. **A**, note how the relative proportion of *wnt8* expression in the telencephalon (dotted white line) grows from left to right. **B**, the amount and extent of *foxg1* expression is less in non-mbuna and treated mbuna versus mbuna and treated non-mbuna. The PSB will form one stage later, approximately where *wnt8* and *foxg1* meet.

4.3.3 PSB Position can be manipulated via WNT and Hh redeployment

We then sought to artificially manipulate the WNT and Hh pathway in both mbuna and non-mbuna in order to determine which pathway is integral for PSB placement. Treatment of mbuna embryos with cyclopamine, a Hedgehog antagonist, for a brief window during neurula, knocks down *shh* and slows its progression dorsally in early somitogenesis (mid-right panels of Figure 4.2A and B); which phenocopies *shh* expression in non-mbuna. In the converse, upregulation of *shh* via SAG during neurula in non-mbuna embryos causes an expansion of *shh* in early somitogenesis much like mbuna embryos (mid-left panels, Figure 4.2A and B).

Table 4.1 Differences between cichlid lineages in the pallial/subpallial compartments of the telencephalon. Data shown is percentage of the total telencephalon occupied by the pallium (%Pal) and subpallium (%SPal). Values represent the pooled mean of several individuals in three species of mbuna and three species of non-mbuna with ± 1 standard deviation. Percentages are components of the same whole, thus st. dev. is the same for both, indicated by the asterisk. Data was measured from scaled frontal images of embryos from stages 13 to 15 (see Figure 4.1).

	% Pal	% SPal
Mbuna (N=16)	39.0 ± 4.0	61.0 ± 4.0
$Mbuna_{DMSO}$ (N=13)	38.7 ± 3.9	61.3 ± *
$Mbuna_{Cyclo}$ (N=17)	58.4 ± 3.4	41.6 ± *
$Mbuna_{LiCl}$ (N=14)	57.6 ± 2.7	42.4 ± *
$NMbuna_{SAG}$ (N=11)	40.6 ± 5.0	59.4 ± *
$NMbuna_{IWR}$ (N=9)	38.4 ± 5.9	61.6 ± *
$NMbuna_{DMSO}$ (N=8)	56.3 ± 1.8	43.7 ± *
Nmbuna (N=16)	58.3 ± 2.1	41.7 ± *

Notably, treatment of mbuna embryos with LiCl, a WNT agonist, phenocopies both the cyclopamine results and non-mbuna embryos, and the reverse, treatment of non-mbuna with the WNT antagonist IWR-1, emulates SAG-treated non-mbuna and mbuna embryos (Figure 4.2A and B). This indicates that the Hh and WNT pathways inversely affect one another; which is of interest because although there are many downstream genes mediate the two pathways, there is no known direct, antagonistic relationship between the Hh and WNTs. In addition, both upregulation of Hh (SAG treatment on non-mbuna) and downregulation of WNT (IWR-1 treatment) are sufficient to induce *foxg1* earlier, as seen in the mbuna, and the opposite pathway manipulations in mbuna embryos can reproduce the *foxg1* delay observed in non-mbuna (Figure 4.2C).

Finally, we investigated the effect of the treatments on the proportion of the pallial/subpallial compartments of the telencephalon. Our data show that manipulation of either Hh (cyclopamine treatment *vs.* DMSO controls, t=14.468, p<<0.001; SAG vs. DMSO, t=9.574, p<<0.001) or WNT (LiCl vs. DMSO, t=14.540, p<<0.001; IWR *vs.* DMSO t=8.629, p<0.001) pathways is sufficient to change the pallial/subpallial proportion from mbuna values to non-mbuna percentages and vice-versa (Figure 4.1B, C; Table 4.1 shows percentages for neurula-treated mbuna and non-mbuna). This indicates a shift in the placement of the PSB boundary via differential deployment of Hh and WNT signal.

This data suggests that mbuna and SAG/IWR-treated non-mbuna possess telencephala that have been 'ventralized' by Hh signal acting through *foxg1* to block the dorsal WNT factors (Figure 4.3A and B, two left panels). By contrast, non-mbuna and cyclopamine/LiCl-treated mbuna possess telencephala that have been 'dorsalized' by the WNT signal that precedes *foxg1* expression (Figure 4.3A and B, two right panels), which results in a shift in the PSB boundary observed later in ontogeny (Figure 4.1). There may also be a mediating factor that is positively regulated by the WNT pathway, restricts the Hh pathway, and is a dorsal analog to the function of *foxg1.*

4.4 Discussion

Our study describes a shift in the placement of the PSB between two ecologically and phylogenetically distinct lineages of Lake Malawi cichlid. Mbuna cichlids possess a PSB that is shifted dorsally relative to non-mbuna, which results in a larger subpallium in mbuna, and a larger pallium in non-mbuna. As it has been previously demonstrated in cichlids (Sylvester *et al.* 2010), a shift in a major patterning boundary could have important implications in the long-term development of the brain, including within the telencephalon. We explain these findings by examining a gene circuit involving *shh, foxg1*, and *wnt8* that is ultimately responsible for the formation of the PSB (Danesin *et al.* 2009). *in situ* hybridization data in cichlids suggests that this gene circuit functions the same as in zebrafish, but also exhibit heterochrony in both the DV deployment of *shh* and the establishment of *foxg1* expression. Assuming the genes interact in cichlids as they do in zebrafish, this heterochrony should be sufficient to shift the PSB.

To test this hypothesis, we artificially up- or downregulated both the Hh pathway (via SAG or cyclopamine, respectively) and the WNT pathway (LiCl or IWR) and investigated how manipulation affects PSB placement. As expected, chemical treatments changed PSB position in both mbuna and non-mbuna, and Hh downregulation in mbuna delayed the onset of *foxg1* in mbuna, phenocopying non-mbuna. We discovered a novel interaction between the WNT and Hh pathways; up or downregulation of WNTs suppresses *shh* in the DV axis, which in turn slows or quickens the onset of *foxg1* expression. We postulate that

another factor must integrate the two pathways, positively upregulated by WNTs, working from the dorsal presumptive telencephalon.

4.4.1 gli3 as a candidate for WNT-mediated suppression of the Hh pathway

The factor working with the WNT pathway to suppress the Hh pathway must fulfill several criteria. It must: i) be able to block Hh expression and be positively associated with WNTs, ii) be expressed at the appropriate temporal and spatial location and iii) be involved in specification of the telencepahlon. We have searched the literature and carried out ISH analysis to screen a list of possible candidates. *gli3* is a prime candidate to fulfill this dorsal mediator role.

First, *gli3* functions as a transcription factor in the Hh pathway. Gli3 protein is cleaved into an N-terminal form (gli3R), which in turn represses Hh signaling in the brain (Blaess *et al*. 2006). Cleavage of Gli3 is blocked by strong Hh signaling, resulting in a weak activator form (gli3A) that serves as an effector of the Hh pathway. This sets up an important, well-described, gradient-based interaction between Hh signal and *gli3* that establishes the DV axis in the neural tube (Altaba *et al.* 2003, Fuccillo *et al.* 2006, Ulloa and Martí 2010). In addition, Gli3 is a positive regulator of WNT signaling in the forebrain (Fotaki *et al*. 2011), and WNT signaling is a positive regulator of specifically the repressor form of Gli3 (Alvarez-Medina *et al*. 2008; Li *et al*. 2009).

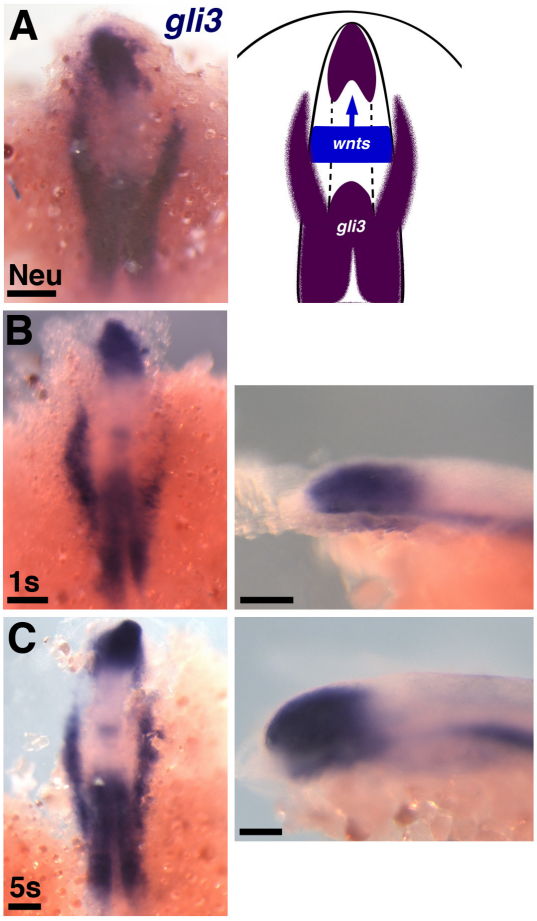

Figure 4.4. *gli3* expression is set between the competing WNT and Hh pathways. All images are of the non-mbuna, *Aulonocara jacobfrebergei.* Neu = neurula, 1s and 5s refer to the number of somites. Scale bars are 100 um. **A** shows a dorsal image of *gli3* expression at the time of treatment (left panel) and a schematic depicting *gli3* expression and the progression of WNT signal as ontogeny continues (right panel). **B** and **C** show a dorsal (left) and lateral (right) view of *gli3* expression as it clears from both the diencephalon and ventral forebrain.

Second, *gli3* is expressed at the appropriate space and time in cichlid embryos (Figure 4.4). *gli3* expression appears in the neural plate as Hh expression begins to enter the plate ventrally during neurula (Figure 4.4A). At this point *gli3* marks the entire presumptive forebrain, and is coexpressed with *shh.* As ontogeny progresses into somitogenesis, *gli3* expression begins to fade from the ventral forebrain and diencephalon (Figure 4.4B) and eventually clears the diencephalon; it continues to clear from the ventral forebrain, and coalesces in

the presumptive telencephalon (Figure 4.4C). By stage 11, *gli3* is coexpressed with *foxg1*, and flanked by *shh* expression ventrally and *wnt8* expression posterior. One stage later, as the presumptive PSB is being set, *gli3* is only expressed in the presumptive pallium (data not shown). Thus, *gli3* fulfills the criteria of persistent expression across all relevant stages of telencephalic development.

Third, *gli3* is necessary for proper specification of the pallium. Loss of Gli3 in the mouse results in misexpression of WNTs in the presumptive pallium, which fails to develop properly (Thiel *et al.* 1999, Thiel *et al.* 2002). In fact, DV specification of the telencephalon as a whole involves mutual repression between Hh and *gli3* (Rallu *et al.* 2002, Hébert and Fishell 2008), and *gli3* may be responsible for the first induction of WNT expression in the presumptive pallium (Mullor *et al.* 2001, Thiel *et al.* 2002).

Thus, we propose a model in which *gli3* acts a dorsal mediator analogous to *foxg1*: *foxg1* is downstream of Hh, can repress the WNT signal and specifies subpallial fate; *gli3* is downstream of WNT, can repress the Hh signal and specifies pallial fate (Rallu *et al.* 2002, Hébert and Fishell 2008; Ulloa and Martí 2010).

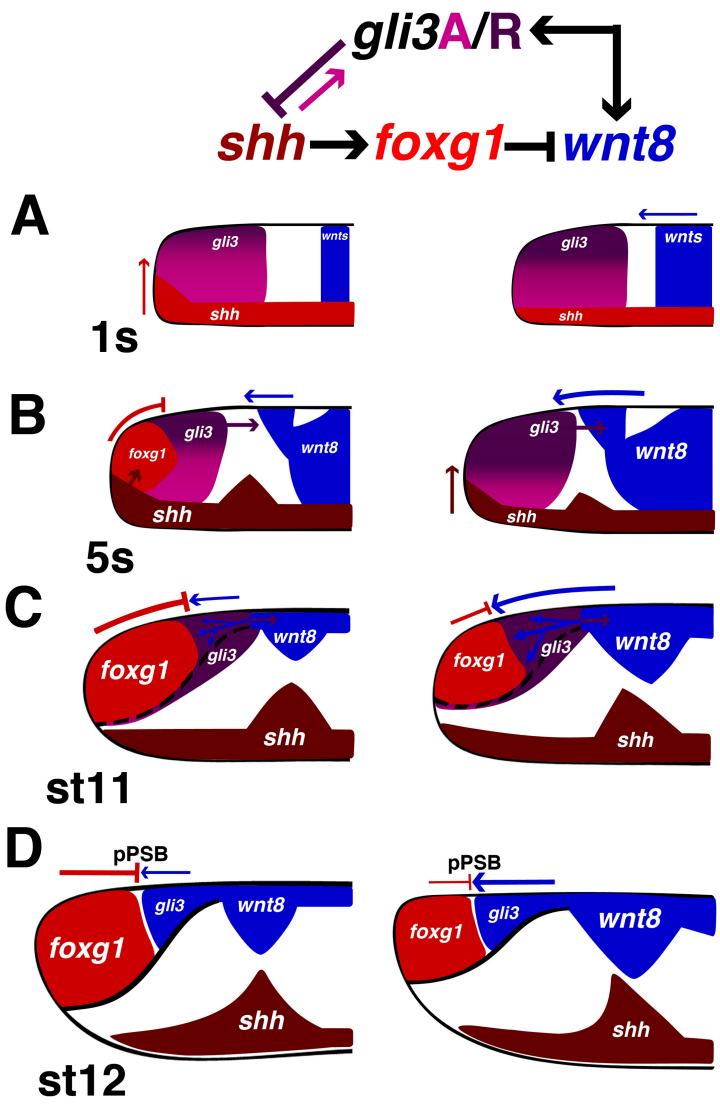

Figure 4.5. The PSB is set by competing Hh and WNT pathways, mediated by *foxg1* and *gli3*. The top of the figure shows the gene network that works to set PSB position. Arrows indicate positive signal; T-bars are blocks to expression. The colored interactions between *shh* and *gli3* reflect the opposite relationship between the two protein forms of *gli3*. Colors in network correspond to expression colors in model. The DV gradient from purple to pink represents the *gli3* R/A protein ratio. Mbuna is on the left, non-mbuna is on the right side. All images are lateral, midline schematic representations. **A** shows a higher DV progression of *shh* expression in mbuna versus a heterochronic deployment of WNTs in non-mbuna. Increased *shh* signal in mbuna leads to an earlier inducement of *foxg1* in **B**, whereas non-mbuna *shh* is just starting to move dorsally. However, non-mbuna WNT expression is driving the formation of more gli3R protein, which forms at a slower rate in mbuna. **C**, as *wnt8* begins to move into the tel, mbuna *foxg1* is expressed in a much larger domain, and is able to block *wnt8* to a greater degree than in non-mbuna. **D**, the presumptive PSB forms at the interface of *wnt8* and *gli3* on one side, and *foxg1* on the other.

4.4.2 A Model: PSB position is set by a time sensitive interplay between shh, foxg1, gli3, and wnt8

The DV shift in PSB position between mbuna and non-mbuna cichlids could be the result of the heterochrony of *shh* and *foxg1* in mbuna and the previously published WNT heterochrony in non-mbuna (Sylvester *et al.* 2010). *gli3* and the ratio of its Hh induced activator form (gli3A) versus the WNT promoted repressor form (gli3R) serves as the mediator between the two timing differences. In our model, there are thus two major differences between mbuna and non-mbuna, both involving competing Hh and WNT signaling. The mbuna telencephalon expresses *shh* more strongly in the anterior portion of the tube (hence a higher gli3A/gli3R ratio) and *foxg1* is concomitantly expressed earlier; *foxg1* in turn represses the dorsal WNT signal as soon as it enters the presumptive telencephalon (Figure 4.5A-C, left side). The non-mbuna telencephalon is more strongly affected by the anterior WNT signal described by Sylvester *et al* (2010). This produces a stronger gradient of gli3R/gli3A, which represses Hh signal, reducing its ability to induce *foxg1* expression, which allows *wnt8* to permeate through a larger relative portion of the presumptive telencephalon (Figure 4.5A - C, right side). These differences reinforce one another in subsequent stages due to the roles *foxg1* and *gli3* serve in telencephalon development to produce mbuna with relatively larger (ventral) subpallia and non-mbuna with relatively larger (dorsal) pallia (Figure 4.5D).

Treatment of mbuna and non-mbuna with Hh and WNT agonist/antagonists illustrate the strict temporal order of these events. As already described by

Danesin *et al.* (2009), *foxg1* only needs to be induced by *shh,* then it becomes self-sufficient. Cichlids corroborate this claim as well, as treatment of mbuna with cyclopamine after neurula does not change the timing of *foxg1* expression or the position of the PSB. However, this same temporal specificity applies to non-mbuna as well; SAG treatment during early somitogenesis does not induce *foxg1* more quickly, nor does it change the PSB (data not shown). This implies that if *shh* is not increased by the end of neurula, another factor, gli3R in our model, prevents additional Hh signal from inducing foxg1 more quickly.

WNT treatments shed light on why this is the case; WNT pathway upregulation (LiCl) in mbuna consistently phenocopies non-mbuna throughout neurula into early somitogenesis, as long as treatment occurs before the induction of *foxg1* at 5s. However, the effect of downregulation of WNTs (IWR) in non-mbuna on PSB position diminishes if treatment occurs after neurula (data not shown). Another way this can be interpreted is an increase of WNT prior to telencephalon specification can restrict the Hh pathway, but there is only a short period of time the Hh pathway is unencumbered by WNTs. Our model predicts, and other studies have shown (Wen *et al.* 2010, Besse *et al.* 2011) that the activator form of *gli3*, gli3A, decreases and the repressor form, gli3R, increases over ontogeny. Gain of WNT (in non-mbuna embryos or LiCl treated mbuna) will directly increase the production of more gli3R, suppress Hh and subsequently delay the induction of *foxg1*, but loss of WNT (in mbuna embryos or IWR treated non-mbuna) is only effective if the gli3R/gli3A ratio is low enough to allow Hh expansion.

Therefore PSB position can be viewed as the outcome of competition between the Hh and WNT pathways for influence on the DV axis. In mbuna embryos, Hh is stronger, *foxg1* is deployed more quickly, and is able to rapidly reduce the influence of WNTs dorsally. In non-mbuna, WNTs moves more quickly along the AP axis, gli3R accumulates more quickly, which in turn confines Hh ventrally.

4.4.3 PSB position reflects an evolutionary difference in the deployment of genes working along the AP and DV neuraxes

The placement of the PSB on the DV axis of the telencephalon allocates more cells in the subpallial compartment in mbuna, and more to the pallial compartment in non-mbuna, which can impact the specification of neuronal subtypes and arealization of the telencephalon later in ontogeny (Herbert and Fishell 2008, O'Leary and Sahara 2008). This leads us to postulate how differential specification of telencephalic compartments is of ecological significance to adult mbuna and non-mbuna. An expansion of the subpallial compartment can directly lead to larger olfactory bulbs, as is observed in many mbuna species (van Staaden *et al.* 1994, Huber *et al.* 1997) as well as an increased ability to process the complex environmental and social interactions involved in life on the rocky reefs of the Rift Lakes (Shumway 2008, Gonzalez-Voyer *et al.* 2009). The dorsal pallium is responsible for interpreting visual signals (Sur and Rubenstein 2005), which would be of selective benefit to many vision-oriented non-mbuna species. Therefore, what this DV shift of the PSB can also

be indicative of is a developmental choice between an elaboration of vision-associated structures on the AP neuraxis versus smell and taste on the DV neuraxis (Sylvester *et al.* 2011). Mbuna ultimately will have brains suited for a rocky habitat, with a de-emphasis on vision and a reliance on smell and taste to search for food (Huber *et al.* 1997, Sylvester *et al.* 2010). This requires an elaboration of ventral areas of the brain, achieved by an expansion of Hh expression, not unlike what has been previously described in the cavefish, *Astyanax mexicanus.* Just as a loss of eyes, an expansion of ventral forebrain, an elaboration of the olfactory bulbs and an increased number of tastebuds are morphological consequences of an initial increase of Hh expression in cavefish (Menuet *et al.* 2007, Rétaux *et al.* 2008, Yamamoto *et al.* 2009, Pottin *et al.* 2011), so is the position of the PSB in mbuna cichlids. Thus, the expanded pallium of non-mbuna can be seen as a consequence of increased WNT activity along the AP neuraxis, which would affect all structures under the influence of WNT signal (Sylvester *et al.* 2010, 2011). Hence the difference in PSB position between mbuna and non-mbuna cichlids is not limited to the specification of the telencephalon, but rather a morphological marker of an early ontogenetic decision to change the layout of the entire brain (Sylvester *et al.* 2011).

4.4.4 Conclusions

In this study, we document a DV shift in the position of the PSB between mbuna and non-mbuna cichlids. We demonstrate, using temporally precise small molecule treatments that target the Hh and WNT pathways, that a gene circuit

involving *shh, foxg1, wnt8* and possibly *gli3* is responsible for PSB variation in cichlids. We offer a new model in which Hh and WNTs compete for influence in the anterior neural tube, mediated by *foxg1* on the Hh side and *gli3* on the WNT; the PSB forms at the interface between these two pathways. An important aspect of *gli3* involvement in our model is the ratio between two post-translational forms, gli3A and R, which we are not able to investigate via *in situ* hybrization. Additional experimentation will be needed to confirm if there is a difference in gli3A/R ratio between mbuna and non-mbuna, and if an increase of Hh or WNT signal can increase the amount of gli3A or gli3R, respectively. Another aspect to consider is what role that the other two signal pathways involved in telencephalon specification, BMPs and FGFs, play in our model of PSB variation. BMPs work alongside WNTs in specification of the dorsal telencephalon (Ohkubo *et al.* 2002), and may also be positively regulated by *gli3* (Thiel *et al.* 2002). FGFs, particularly *fgf8*, is induced by *shh*, but in turn negatively regulates Hh signal in the telencephalon, much like *gli3; foxg1* is a target of FGF signal, and *fgf8* has already been shown to be expressed earlier in eyeless versus eyed forms in the cavefish (Pottin *et al.* 2011). Finally, due to the correlative nature of our experiments, we cannot verify the molecular basis of the PSB shift in cichlids, only link it to what has been demonstrated in zebrafish. Nevertheless, our analysis shows that competition for influence between the Hh pathway ventrally and the WNT pathway dorsally is responsible for variation in the position of the PSB in cichlids.

4.5 Materials and Methods

4.5.1 Embryonic telencephalon measurements

Areas of pallial and subpallial compartments were measured in frontal section(s) of the telencephalon of mbuna and non-mbuna species. Mbuna species measured include *Cynotilapia afra* (CA), *Labeotropheus fuelleborni* (LF) and *Maylandia zebra* (MZ); non-mbuna species include *Aulonocara jacobfreibergi* (AJ), *Copadichromis borleyi* (CB) and *Mchenga conophorus* (MC). Measurements were made starting at the first developmental stage (stage 13, Sylvester *et al.* 2010) where the pallium and subpallium can be distinguished through cellular restriction/movements around the PSB and each subsequent stage until telencephalon eversion (stage 16). In addition, placement of the PSB was confirmed using gene expression: the pallium is marked by *pax6* or *emx3* expression and the subpallium is marked by *dlx2* or *isl1* expression.

Measurements were made using ImageJ with scaled images of frontal sections of embryos, and landmarks were used such as the anterior commissure ventrally and the epithalamus dorsally in order to maintain consistency among individuals. Between 10 and 20 individuals per species were selected and sectioned, and individuals with oblique sections were eliminated, leaving 4-6 individuals for measurement. Each pallial and subpallial measurement was expressed as a percentage of the total telencephalon. As ontogeny progressed, the telencephalon encompasses more than one frontal section. When necessary, the sum of the area of both the pallium and subpallium across two or more

sections (Cavalieri method, Σ [area of each section] * [distance between sections]) was used to generate percentages.

4.5.2 in situ hybridization

in situ hybridization (ISH) experiments are based on previously published protocols (Fraser *et al* 2008). Probes were constructed from cDNA sequences derived from partial genome assemblies of Lake Malawi cichlids (Loh *et al* 2008). The RNA probes can be used on any of the cichlid species examined in the study; Lake Malawi cichlids genetic variation is comparable to laboratory strains of zebrafish (Loh *et al* 2008). All ISH experiments were performed with multiple specimens (multiple individuals within single broods, then repeated at least twice with alternative broods) to fully characterize expression patterns within and across species. Once ISH was complete, embryos were embedded in gelatin-albumin blocks and sectioned as in Sylvester *et al* 2010.

4.5.3 Chemical treatments

Embryos from three mbuna species, *Labeotropheus fuelleborni* (LF), *Maylandia zebra* (MZ), and *Cynotilapia afra* (CA), were treated with LiCl, which upregulates the WNT pathway, or cyclopamine, which downregulates Hh, in order to phenocopy the PSB position observed in non-mbuna species. LiCl upregulates WNTs by inhibiting GSK3, which normally degrades β-catenin, an effector of the WNT pathway. The LiCl stock solution and working concentration were made via methods described in Sylvester *et al.* (2010). Cyclopamine

inhibits the Hh pathway by binding and inactivating the Hh receptor, Smoothened (Smo). A 5 mM cyclopamine stock was made by dissolving 21 mg cyclopamine (LC Laboratories) into 10 mL of Dimethyl Sulfoxide (DMSO); to obtain the final working concentration of 50 uM, 70 uL of this stock was diluted into 7 mL fish water.

Next, a complementary experiment was done using non-mbuna species, *Aulonocara jacobfreibergei* (AJ)*, Copadichromis borleyi* (CB), and *Mchenga conophorus* (MC), in which embryos were treated with a *shh* agonist, SAG (Enzo Life Sciences), or a WNT antagonist, IWR-1 (Enzo), in order to phenocopy mbuna PSB. SAG agonizes Hh activity by activating Smo, and IWR induces WNT activity by directly binding to Axin2, which stabilizes and allows the Axin complex to destroy β-catenin. Stock and working concentrations for both SAG and IWR are 100 uM and 1uM respectively. The stock solution for both treatments was made by dissolving 0.5 mg SAG into 5 mL of DMSO (1 mg into 10 mL for IWR) to get the stock. Working concentrations for both chemicals were obtained by dissolving 70 uL of stock into 7 mL fish water.

Table 4.2. Overview of Chemical Treatment Experiments. The table summarizes, from left to right, the chemicals used in the treatment experiments, how the stock solution and working concentrations were made, and the effect of treatment on embryos. DMSO = dimethyl sulfoxide, WNT = wingless gene pathway, Hh = hedgehog gene pathway, M = molar, L = liter, m = milli, u = micro.

Chemical Name	Stock Solution	Working Concentration	Treatment Effect
Lithium Chloride	640 mg into 15 mL DMSO (4M)	8.75 uL stock into 7 mL fish water (5mM)	Agonizes WNT by increasing β-catenin
Cyclopamine	21 mg into 10 mL DMSO (5mM)	70 uL stock into 7 mL fish water (50uM)	Antagonizes Hh by inactivating Smoothened
IWR-1	1 mg into 10 mL DMSO (100uM)	70 uL stock into 7 mL fish water (1uM)	Antagonizes WNT by stabilizing Axin2
SAG	0.5 mg into 5 mL DMSO (100uM)	70 uL stock into 7 mL fish water (1uM)	Agonizes Hh by activating Smoothened

Embryos in broods (one brood consists of 20 to 40 individuals) from each species of mbuna and non-mbuna were treated during the neurula stage (approximately 36 to 40 hours post fertilization) for 5 hours at 28°C, and bathed with either a chemical, 0.125% (control for LiCl) or 1% (for cyclopamine, SAG, and IWR) DMSO vehicle controls. This experiment was repeated for 2 to 4 additional broods for each species. After treatment, embryos were washed twice with fish water, placed in culture flasks with fresh fish water, and incubated at 28°C until they reached the desired stage. Treatment and control embryos were post-processed (ISH, sectioning, measurements) identically to descriptions above for untreated embryos.

4.6 Acknowledgements

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CHAPTER 5

OVERALL CONCLUSIONS

Comparisons of brain morphology across an expanding group of vertebrates continue to provide insight into brain evolution (Iwianuk and Hurd 2005, Reep *et al.* 2007, Mueller and Wullimann 2009, Charvet *et al.* 2009, Yopak *et al.* 2010). However, it is becoming clear that a deeper understanding of how brain patterning drives evolution is needed to fully understand how brain diversity arose in vertebrates. Recently, an increasing number of studies have been looking at the developmental aspects of brain evolution across a variety of vertebrates (Menuet *et al.* 2007, Charvet and Striedter 2008, 2010, Sylvester *et al.* 2010, McGowan *et al.* 2010, Pottin *et al.* 2011). Of the vertebrates described in these new brain evo-devo studies, cichlids are becoming an excellent system to study the role of patterning on brain evolution, due to well-described differences in brain morphology linked with ecology and behavior (van Staaden *et al.* 1995, Huber *et al.* 1997, Pollen *et al.* 2007, Shumway 2008, Gonzalez-voyer *et al.* 2009, Sylvester *et al.* 2010), coupled with the ease at which to conduct *in situ* hybridization based surveys for slight modifications of well described gene pathways responsible for large variations in adult morphology (Albertson *et al.* 2005, Fraser *et al.* 2008, Sylvester *et al.* 2010, Roberts *et al.* 2011). For these reasons, cichlids offer a unique opportunity to study aspects of brain evolution that is not possible with other commonly used model organisms.

This dissertation uses the cichlid system to investigate the role of patterning (e.g. early developmental events that work to specify the brain) in vertebrate brain evolution. Classically, neurogenesis (e.g. the process by which undifferentiated neural precursors become functional neurons) has been cited as the driving force behind brain evolution (Finlay *et al.* 2001), and I do not seek to invalidate that claim. Instead I use cichlids to illustrate that patterning has a role to play in brain evolution as well; a role that is lost in evolutionary time when doing broad scale comparisons of brain morphology across vertebrates.

In chapter 2, I presented a brief overview of the two accepted modes of brain evolution, developmental constraint and mosaic evolution, and described how, due to the methods used to elucidate these theories, both are explained via differential onset of neurogenesis. I used recent studies done in fish, primarily in the cavefish *Astyanax mexicanus* and in the East African cichlids, to formulate a model that described how patterning can work across major developmental axes in the presumptive brain (e.g. neuraxes) to generate brain diversity. I presented two alternate sensory-based modalities the brain can take, visual or smell/taste optimized. Visual brains can be generated via changes to gene pathways (WNTs) working along the AP neuraxis whereas smell/taste brains derive from changes to pathways on the DV neuraxis (BMPs, FGFs, Hh). I then reinterpreted the developmental constraint and mosaic evolution theories of brain evolution in terms of patterning. The crux of these models is still the same; difference in the timing of developmental events drives brain variation (e.g. late equals large) but rather than a delay in the onset of neurogenesis, I postulated that a delay in the

deployment of one gene pathway working along one neuraxes versus another also drives brain variation. If the change is early enough, there is a widespread change in brain morphology, fitting the constraint model of brain evolution. However, as major developmental boundaries begin to subdivide the brain further, changes to gene pathways have a more targeted effect, resulting in the mosaic model. Finally I ended the chapter by repeating that brain evo-devo cannot be focused on one developmental process, be it neurogenesis or patterning, but must encompass all of brain ontogeny.

Chapter 3 is a study of how a change to the WNT pathway working along the AP neuraxis can generate brain diversity in cichlids. We first demonstrated that cichlids exhibit a huge range of adult brain morphology, comparable to the amount of variation over seven mammalian taxa, notable because cichlids achieved this variation in only a few million years, versus 150 million years in mammals. We then found that this variation is apparent in embryonic brains as well, via cerebrotype measurements of the forebrain. This variation consisted of a split between mbuna, which have large telencephala, and non-mbuna, which have large thalami. The developmental cause for this difference was an AP shift in the position of a major signaling boundary, the zona limitans intrathalamica (ZLI). The ZLI in non-mbuna is more anterior than in mbuna, driven by a faster expansion of WNT signal from the midbrain-hindbrain boundary (MHB), resulting in a greater proportion of the forebrain being patterned for thalamus rather than telencephalon. This rapid expansion of WNT can be replicated in mbuna embryos when they are bathed with a WNT agonist, LiCl, which results in both an

anterior shift of the ZLI, and expansion of the thalamus. Finally, we ended the study with the discovery of a non-synonymous SNP, alternatively fixed between mbuna and non-mbuna, in the *irx1* gene. *irx1* has a positive feedback loop with the WNT pathway, which led us to postulate that this change could be the driving factor behind the rapid deployment of WNTs along the AP neuraxis in non-mbuna.

Chapter 4 described a similar change of a major gene pathway that drives variation between mbuna and non-mbuna; the Hh pathway working along the DV neuraxis. The study investigated the basis behind the shift of another developmental boundary within the developing telencephalon, the pallial-subpallial boundary (PSB). Mbuna have a dorsal shift of the PSB, resulting in a larger subpallium, whereas non-mbuna have a larger pallium. It was found that the underlying cause was a redeployment of genes working in a circuit responsible to positioning the PSB. In mbuna, *shh* is expressed more strongly along the DV axis, *foxg1* is induced more quickly in the presumptive telencephalon, and in turn is able to block *wnt8* as it enters the dorsal portion of the telencephalon. Non-mbuna have a faster deployment of WNTs, and *wnt8* is able to 'grab' a much larger portion of the dorsal telencephalon before *foxg1* is induced to block it. Next, we treated mbuna embryos with an Hh antagonist (cyclopamine) and WNT agonist (LiCl), and treated non-mbuna with the converse, an Hh agonist (SAG) and WNT antagonist (IWR-1), in an attempt to artificially manipulate the Hh and WNT pathways and shift the PSB between mbuna and non-mbuna. Not only were we able to phenocopy the non-mbuna

PSB position in mbuna and vice versa, we also found evidence of an indirect interaction between the Hh and WNT pathways. Just as the Hh pathway can indirectly inhibit the effect of WNT on the telencephalon via *foxg1*, there is an analogous dorsal factor that is downstream of WNTs that can affect the Hh pathway. We used previously published studies and an *in situ* hybridization survey of potential genes to conclude that *gli3* is a likely candidate for this role. Thus, we ended the study by postulating that the position of the PSB is a consequence of the competing actions of the Hh and WNT pathways, mediated by *foxg1* ventrally and *gli3* dorsally.

This dissertation has attempted to identify and elucidate the role of early patterning events on brain evolution. It did this by first presenting a model by which patterning can contribute to the major theories of brain evolution (chapter 2), then presented two case studies focused on either the AP neuraxis (chapter 3) and DV neuraxis (chapter 4), in order to show evidence in support of this model. These studies do not represent the full scope of ways which patterning can drive brain variation; rather they are discrete events in brain ontogeny that evolution has used to generate diversity. Indeed, evolution can act at any point during along the continuum of patterning to neurogenesis, be it early, as this dissertation has described, or later during neurogenesis, as is classically described. Thus, the overall conclusion of this dissertation is that vertebrate brain evolution cannot be fully characterized unless researchers investigate the role of patterning alongside neurogenesis.

5.1 Publications

The following publications represent some of the research conducted during my PhD candidature, arising from the studies reported in this dissertation.

1. Sylvester JB, Rich CA, Loh Yh, van Staaden MJ, Fraser GJ, Streelman JT. 2010. Brain diversity evolves via differences in patterning. *Proceedings of the National Academy of Sciences USA* 107(21): 9718-9723.

2. Sylvester JB, Pottin K, Streelman JT. 2011. Integrated brain diversity along the early neuraxes. *Brain Behavior and Evolution* In press.

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Sylvester JB, Rich CA, Loh YHE, van Staaden MJ, Fraser GJ, Streelman JT (2010) Brain diversity evolves via differences in patterning. *Proceedings of the National Academy of Sciences USA* 107:9718-9723.

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APPENDIX A

SUPPLEMENTARY MATERIALS FOR CHAPTER 3

A1 Supplemental methods

A1.1 Phylogeny of cichlid Irx1

Annotated sequences of zebrafish Irx proteins (ENSDARP00000007800, ENSDARP00000073569, ENSDARP00000016693, ENSDARP00000069381, ENSDARP00000042270, ENSDARP00000051692, ENSDARP00000052337, ENSDARP00000043856, ENSDARP00000045627, ENSDARP00000025947, NP_001001405.1), as well as the Irx1 proteins of *Fugu* (ENSTRUP00000018488, ENSTRUP00000029525), *Tetraodon* (ENSTNIP00000012127), medaka (ENSORLP00000006499, ENSORLP00000017930), stickleback (ENSGACP00000008692, ENSGACP00000011891), and an outgroup from *C. elegans* (C36F7.1), were downloaded from the Refseq (release31) and Ensembl (v50) databases (Pruitt *et al.* 2007, Flicek *et al.* 2008). Together with the cichlid Irx sequence, a multiple sequence alignment was generated using Clustalw (v2.0.10) (Larkin *et al* 2007). Phylogenetic relationships between the gene sequences were determined using various programs of the PHYLIP package (v3.67) (Felsenstein 2007): 1000 bootstrap replicates of the multiple sequence alignment was generated using Seqboot; pairwise distance measurements were calculated using Protdist;

neighbor-joining trees were constructed using Neighbor; the final consensus tree, together with the bootstrap percentages, was generated with Consense.

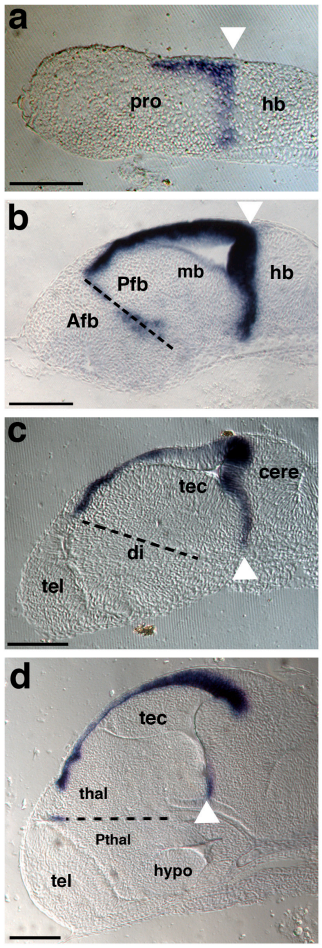

Figure A1. Staging the cichlid brain. A, ISH showing *wnt1* gene expression for *Mchenga conophorus* (MC), at late stage 9. **B**, **C**, and **D** show the same species, gene expression, and layout as **A**. **B**, stage 12. **C**, stage 14. **D**, stage 16. White arrowheads mark the position of the midbrain-hindbrain boundary (MHB); dashed lines mark the position of the zona limitans intrathalamica (ZLI). Scale bars are 100 μm. hb = hindbrain; pro = prosencephalon; mb = midbrain; Afb = anterior forebrain; Pfb = posterior forebrain; cere = cerebellum; tec = tectum; di = diencephalon; tel = telencephalon; thal = thalamus; Pthal = prethalamus; hypo = hypothalamus. All panels are the para-sagittal section with the greatest dorso-ventral extent (hereafter, 'para-sagittal section'), with anterior to the left.

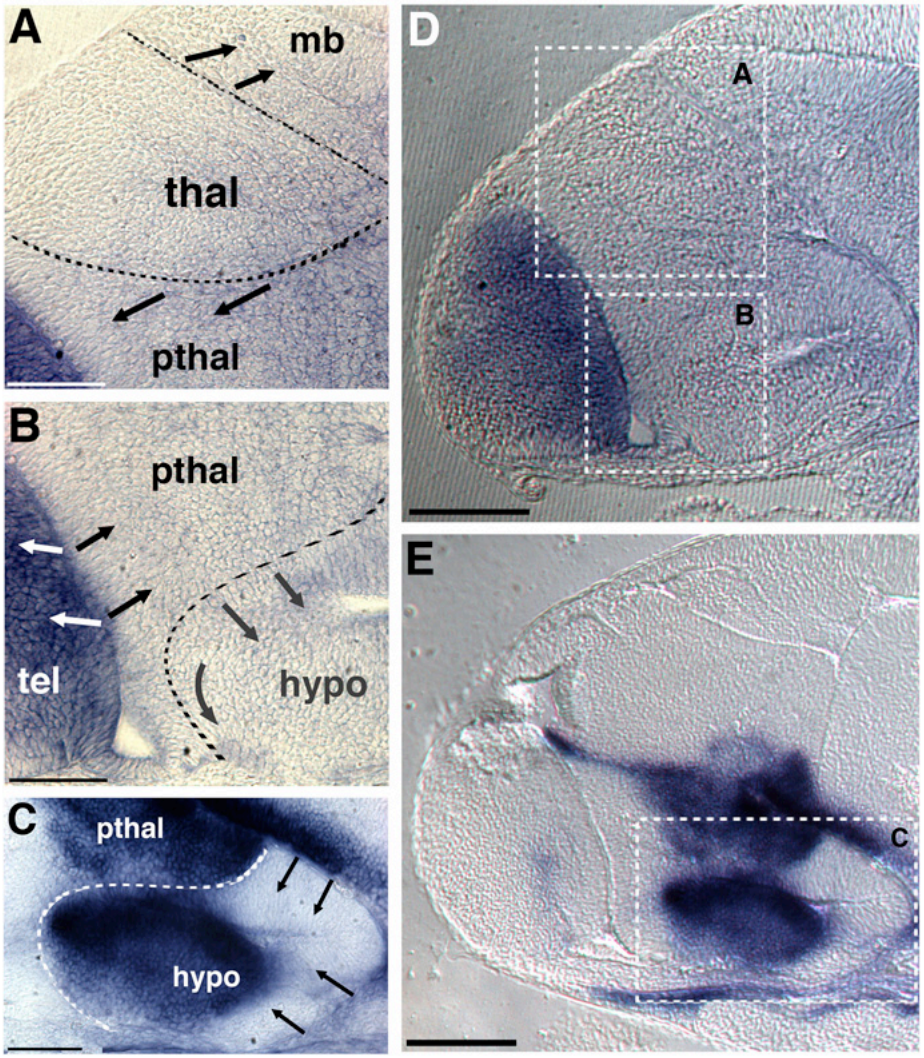

Figure A2. Measuring the embryonic forebrain at stage 16. Panels **A**-**C** are close-up views of boxes **A**-**C** in **D** and **E**. **A** shows the cellular morphology and behavior (marked by arrows) that differentiate the thalamus (thal) from the midbrain (mb) and rest of the forebrain. **B** and **C** show the other three compartments measured in the forebrain, defined both by gene expression (tel in **B** and pthal, hypo in **C**) and cellular behavior (arrows). The dashed lines in **B**, **C** mark the difference between the prethalamus (pthal) and hypothalamus (hypo). **D**, ISH of the gene *foxg1* in the non-mbuna, *Copadichromis borleyi*. *foxg1* differentiates the telencephalon (tel) from the pthal. **E**, ISH of the gene *shh* (*Maylandia zebra*), which marks the hypo, as well as the ZLI, the boundary between the pthal and thal. Scale bars are 50 μm in **A**-**C**, 100 μm in **D**, **E**. All panels are para-sagittal sections with anterior to the left.

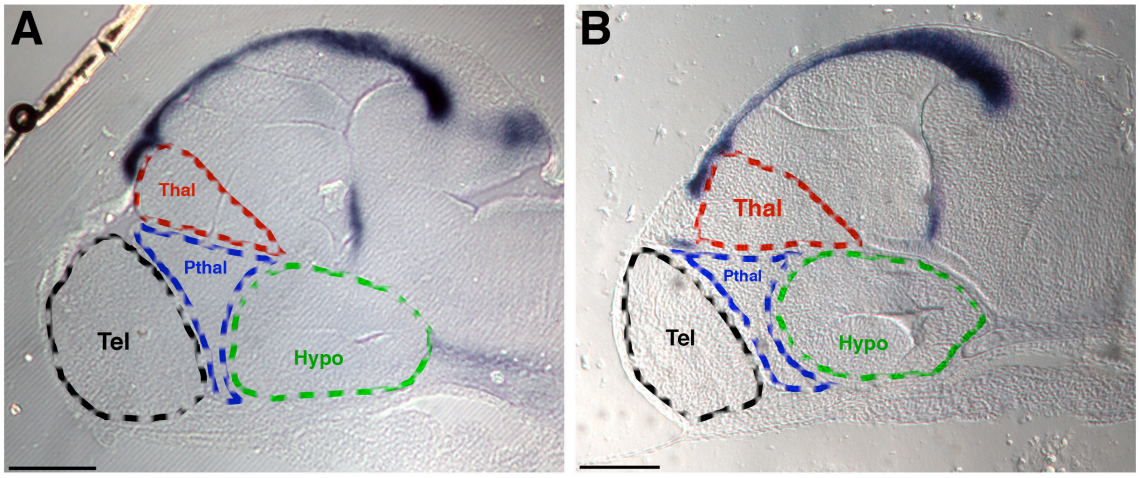

Figure A3. Stage 16 brains differ between mbuna and non-mbuna. A, ISH of the gene *wnt1* in the mbuna, *Maylandia zebra* (MZ). The red (thalamus), blue (prethalamus), black (telencephalon) and green (hypothalamus) dashes outline the four compartments of the forebrain measured. **B**, depicts the same gene expression and dashed outlines as **A**, but in the non-mbuna, *Mchenga conophorus* (MC). The brains of mbuna (**A**) exhibit larger telencephala and smaller thalami than their non-mbuna (**B**) counterparts (Table 3.1). Scale bars are 100 μm. Panels are para-sagittal sections with anterior to the left.

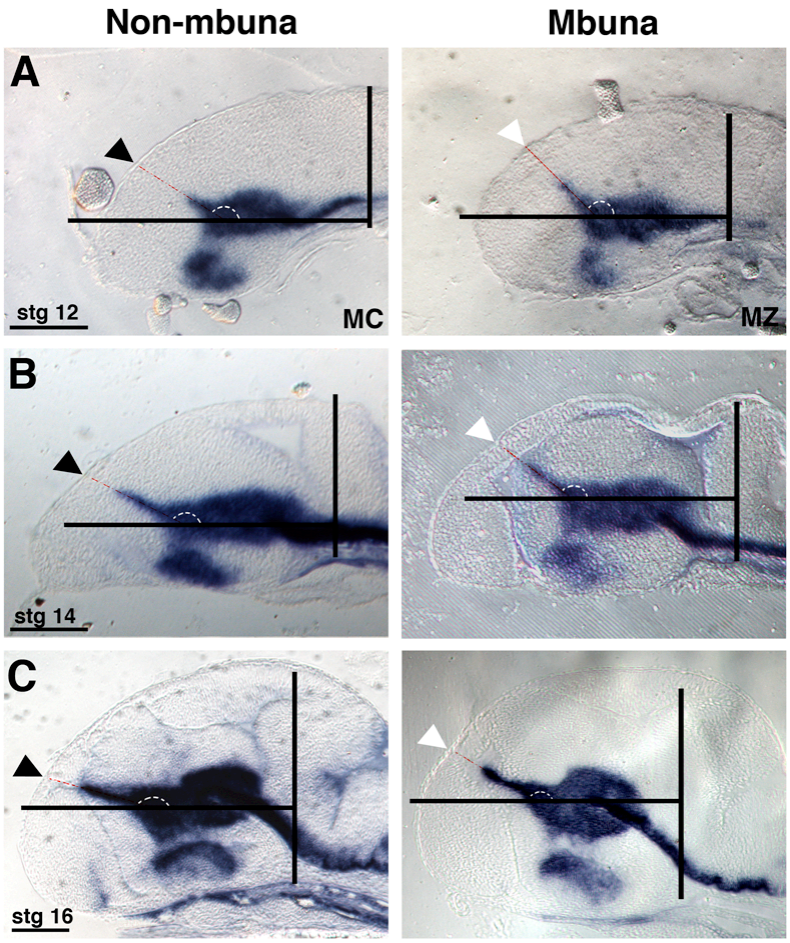

Figure A4. The difference in angle of the ZLI, between lineages mbuna *vs*. non-mbuna, is maintained throughout ontogeny. Rows **A**, **B**, and **C** are ISH for the gene *shh* depicting the angle of the zona limitans intrathalamica (ZLI) across three developmental stages (12, 14, and 16 respectively; see Figure 3). The ZLI is marked by the black arrowhead in non-mbuna (MC), and white arrowhead in mbuna (MZ). The dotted red and white lines show the 'ZLI angle' (see Methods). As development progresses, the initial ZLI wedge narrows into a line and the ZLI angle increases. However, at each stage the average ZLI angle in non-mbuna is greater than that of mbuna (see Figure 3.3). Scale bars are 100 μm. Panels are para-sagittal sections with anterior to the left.

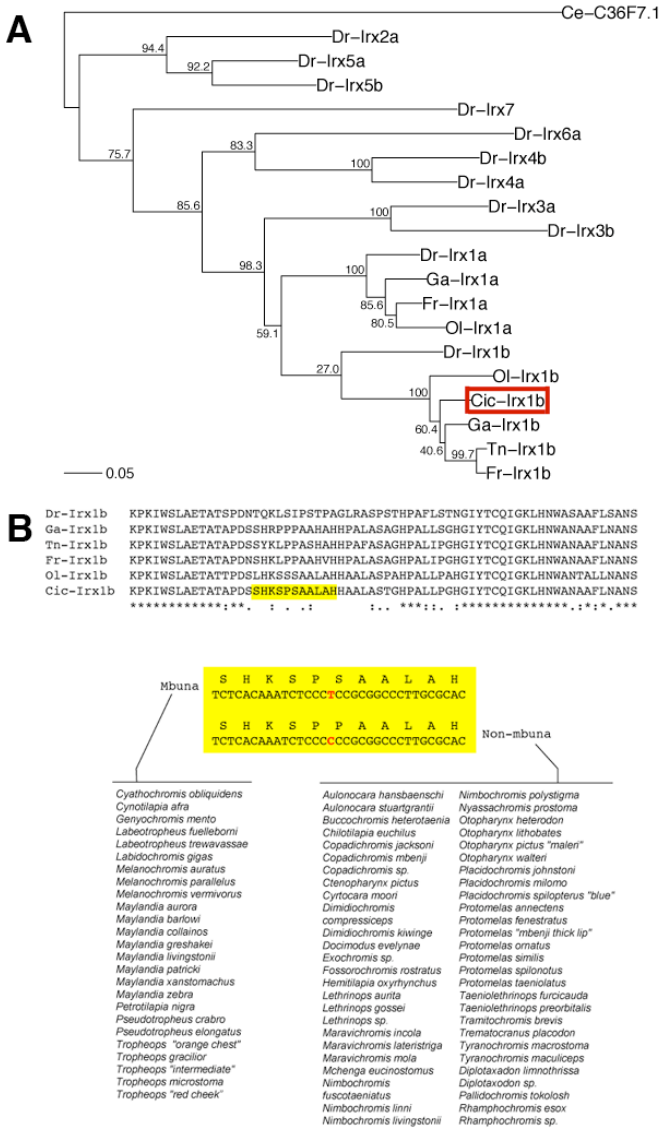

Figure A5. A SNP in *irx1b* is alternately fixed in mbuna *vs*. non-mbuna cichlids. A, Phylogram of fish Irx sequences shows that cichlid Irx1 is Irx1b. The scale bar represents genetic distance. Numbers at nodes are percentages of 1000 bootstrap re-samplings. Ce = *Caenorhabditis elegans*; Dr = *Danio rerio*; Ga = *Gasterosteus aculeatus*; Fr = *Fugu rubripes*; Ol = *Oryzias latipes*; Cic = cichlid; Tn = *Tetraodon nigroviridis*. **B**, local alignment of fish Irx1b amino acid sequences shows the position of the cichlid replacement SNP (yellow, nucleotide is in red) alternately fixed between 25 mbuna and 52 non-mbuna species.